CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF DIARRHEAGENIC *E. COLI* IN THIKA LEVEL 5 HOSPITAL

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Characterization and antimicrobial susceptibility pattern of Diarrheagenic *E. coli* in Thika Level 5 Hospital

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To (i) my parents, Mr Stephen and Mrs Anne Kanyina for motivating me all through, (ii) my brothers, Moses Maina Kanyina and Benson Muriithi Kanyina for the backing, and (iii) my husband, Simon Magondu Mithamo for the timely support and love.

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LIST OF ABBREVIATIONS AND ACRONYMS

AST	Antibiotic Susceptibility Test
CDC	Centres for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
DAEC	Diffusely Adherent *Escherichia coli*
DEC	Diarrheagenic *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
E. coli	*Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
HIV	Human Immunodeficiency Virus
HUS	Hemolytic Uremic Syndrome
Ipa	Invasion plasma antigen
LEE	Locus for Enterocyte Effacement
LT	Heat Labile enterotoxin
PCR	Polymerase Chain Reaction
PIN	Participant Identification Number
QiaDNA	Qiagen DNeasy DNA extraction protocol for bacterial cultures
STEC	Shiga-toxin producing *Escherichia coli*

ST	Heat stable enterotoxin
TCBS	Thiosulfate Citrate Bile salts Sucrose
UNICEF	United Nation Children's Fund
US	Unites States
VTEC	Verocytoxin producing *Escherichia coli*
WHO	World Health Education
XLD	Xylose Lysine Deoxycholate agar

LIST OF DEFINITION OF TERMS

Adhesion: These are components of the surface of the cells that enable bacteria to attach to others cells or surfaces.

Antibiotics: Drugs derived from bacterial sources and are used to treat or prevent bacterial infections.

Antimicrobial resistance (AMR): Resistance of a microbe to a drug which it was earlier sensitive (WHO, 2013).

Chromosomal Pathogenicity Island: These are distinct genetic elements encoding virulence factors of pathogenic bacteria. They belong to the class of genomic islands, which are common genetic elements sharing a set of unifying features. Genomic islands are acquired by horizontal gene transfer and various diverse types have been isolated in pathogenic and non-pathogenic bacteria (Ohad Gal-Mor and B. Brett Finlay, 2006).

Commensals: These are two different species living together harmoniously without harming each other but benefiting each other. Some bacteria in the human digestive tract aid in food processing and production of different types of vitamin B which are essential for normal human health without causing harm to the human body (normal flora).

Efficacy: It is the ability of an intervention or drug to produce a desired effect.

Enteric bacteria: These are rod-shaped Gram-negative bacteria; most occur as normal intestinal commensals or as disease causing agents in the intestines of humans and other animals.

Enteropathogen: A microorganism that causes disease of the intestine.

Enterotoxin: A toxin that is produced by bacteria targeting the intestinal mucosal membrane and leads to diarrhea and vomiting observed in food poisoning patients.

Genes: The molecular unit of heredity of a living organism.

Hemolytic uremic syndrome: This is a disorder caused by toxic substances produced after a digestive system infection by *Escherichia coli*

O157:H7. These toxins cause massive damage to the red blood cells resulting to kidney impairment.

Hemorrhagic colitis: This is a self-limiting abdominal condition characterized with (i) abdominal cramps and (ii) bloody diarrhea, without rise in body temperature (fever). It is associated with *E. coli* infection.

Incubation period: This is the interval between acquisition/ ingestion/inoculation of an infectious agent to the development of clinical illness (signs & symptoms): disease onset.

Inflammatory response: This is a reaction generated by the immune system after tissue injury caused by bacterial infection, toxins among other causes. It involves fluid accumulation causing swelling, white cells production which engulfs (i) foreign bodies (micro-organism) and (ii) dead or destroyed cells a collection of which is released as pus.

Informed assent: This is the act of allowing a person under ones care to take part in a research study after getting full information of the study objectives, benefits and risks related to involvement as well as the participants' role in the study. This is usually given by parents or guardians or care-givers on behalf of children under 18 years or persons who at the moment is not in a state to understand the research details.

Informed consent: This is the act of voluntarily accepting to take part in a research study after getting full information of the study objectives, benefits and risks related to involvement as well as the participants' role in the study without undue influence or coercion by a sound 18 years person (adult).

McFarland standards: This is a 0.5 optical density suspension equivalent to approximately 10^8 CFU/ml (CFU = colony forming units) used as a reference to adjust the turbidity of culture and susceptibility inoculating solution.

Minimum inhibitory concentration: This is the lowest concentration of an antimicrobial that is effective to clear a bacterial infection in the blood. This is determined by subjecting bacteria culture to various

antimicrobials concentrations. The lowest drug concentration that inhibits bacterial growth in a bacterial culture is thus termed as minimum inhibitory concentration effective for treatment.

Normal flora: These are bacteria that stay in different parts of the body without causing disease in a normal healthy person. They are commensal and act for mutual benefit to the host.

Oral rehydration therapy: Oral administration of a glucose-based salt solution used in the treatment of dehydration in persons with diarrhea.

Outbreaks: This is the occurrence of cases of disease that exceeds the expected amount of cases in a particular group of persons in a certain region over a given period of time flame.

Pathogenesis: The development of disease; more specifically the cellular events and reactions.

Pathogens: These are disease causing agents and are grouped in defined categories based on similarity of their characteristics such as bacteria, parasites and viruses.

Pathotypes: This is classification of disease causing agent of the same species into distinct groups based on their pathogenicity on a certain host.

Phenotypic information: The observable physical or biochemical features of an organism, which is determined by (i) genetic makeup and (ii) environmental influences.

Plasmid: This is a segment of the DNA independent of the chromosomes that has the capacity to replicate and occurs in bacteria and yeast cells. They contain some genes encoding for proteins, such as enzymes, which carry resistance traits to antimicrobials.

Prevalence: Describes amount of a disease in the population at a certain point in time or in a defined time flame (period).

Prototype: The primitive form; the first form to which subsequent individuals of the class or species conform.

Resistant: This implies that a micro-organism such as a bacterial growth is not inhibited by the minimum effective antimicrobial concentration recommended for treatment.

Surveillance: This is the continuous systematic collection, organization, analysis, interpretation of data and dissemination of derived information for public health action.

Susceptible: This implies that a micro-organism (infection) can be cleared by the minimum antimicrobial concentration (dose) recommended for effective treatment.

Syndromes: This is a collection (group) of signs and symptoms that presents together and characterizes a specific disease.

Vaccine: This is a suspension of (i) dead, (ii) weakened (attenuated) or (iii) modified microorganisms such as viruses, bacteria or rickettsiae that is administered by various routes to boost or produce immunity to a specific disease by provoking the production of antibodies.

Virulence: This is the ability of disease causing organism to produce disease. It is used as a measure of disease severity.

Zoonotic: Diseases that can be passed from animals, whether wild or domesticated, to humans.

ABSTRACT

Diarrheagenic *E. coli* (DEC) are among re-emerging bacterial strains associated with outbreaks of severe diarrhoea and multiple drug resistance. Our objectives were to characterize DEC among diarrheal patients attending Thika Level 5 Hospital and determine their antimicrobial susceptibility patterns. A cross-sectional study was conducted. Consenting patients of all ages seeking diarrhoea treatment at the hospital from April to July 2014 were recruited. A structured questionnaire was used to collect clinical and epidemiological information. Stool samples were collected, inoculated on bacterial differential media for growth of enteric pathogens and antimicrobial susceptibility patterns of DEC isolates determined. Isolates were characterized by Polymerase Chain Reaction for the presence of virulence properties. A total of 402 stool samples were cultured. *E. coli* was isolated from 269, of which 72 (27%) were DEC; 60 (83.3%) enteroaggregative *E. coli* (EAEC), 6 (8.3%) enteropathogenic *E. coli* (EPEC) and 6 (8.3%) enterotoxigenic *E. coli* (ETEC). Of the DEC affected patients, 58% were female, median age was 8 (IQR: 2-28) years, 75% did not boil water and 100% did not treat water. Twenty five (35%) patients with DEC were under-five years of age. Drinking un-boiled water (OR: 2.51, 95% CI: 1.36-4.61) was associated with having DEC. Being under-five years was associated with EAEC ($P<0.05$). Of the 60 EAEC strains, 24 (40%) EAEC isolates were positive for both *aggR* and *aspU* genes, while 36 (60%) were positive for *aspU* gene only. EPEC *eae* (100%, n=6) and ETEC *elt* (100%, n=6). All DEC isolates were sensitive to cefoxitin, meropenem, amikacin, gentamicin and ciprofloxacin. They were resistant to ampicillin (92%), trimethoprim-sulfamethoxazole (92%) and amoxicillin-clavulanic acid (85%). Majority of diarrhea patients were female and predominant DEC strain were EAEC. Drinking un-boiled water was associated with DEC infection. High level of resistance to ampicillin, trimethoprim-sulfamethoxazole and amoxicillin-clavulanic acid were observed. All isolates were sensitive to ciprofloxacin and gentamicin.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Escherichia coli (*E. coli*) is a large group of bacteria, classified as part of the family of *Enterobacteriaceae* that is usually found in the gastrointestinal tract of (i) humans and (ii) other warm-blooded animals. They are transmitted through ingestion of contaminated water or food (faecal oral route) (WHO, 2011). Despite the fact that majority of *E. coli* sub-types are commensals, few emerging strains have the capacity to disrupt normal human gut physiology, causing illnesses ranging from watery diarrhea to deadly hemolytic uremic syndrome. At least six such pathotypes strains have been described: (i) enteropathogenic *E. coli* (EPEC); (ii) enterotoxigenic *E. coli* (ETEC); (iii) enteroinvasive *E. coli* (EIEC); (iv) enterohemorrhagic *E. coli* (EHEC), which is also called shigatoxigenic *E. coli* (STEC); (v) diffusely adherent *E. coli* (DAEC); and (vi) enteroaggregative *E. coli* (EAEC). This classification was based on (i) presence of different chromosomal or plasmid encoded virulence genes which are not found in most commensal sub-types, and (ii) their pattern of interaction with epithelial cells and tissue culture monolayers (Nataro & Kaper, 1998; Todar, 2007; Alikhani *et al.*, 2012; Goldwater & Bettelheim, 2012).

Diarrheagenic *E. coli* cause illnesses through different mechanisms such as (i) adherence, (ii) elaboration of toxigenic mediators, (iii) invasion of the mucus membrane lining the intestines, and (iv) transfer of bacterial proteins into the host cells (Mohammed & Ted, 2002; Hodges & Gill, 2010; Boyd *et al.*, 2014). Diarrheagenic *E. coli* stains are associated with several distinct clinical diarrhea syndromes; (i) childhood and traveler's diarrhea which is associated with enterotoxigenic *E. coli*, (ii) hemorrhagic colitis and hemolytic-uremic syndrome which is linked to enterohemorrhagic *E. coli*, (iii) acute and persistent diarrhea among children and adults which is associated with enteroaggregative *E. coli*, and

(iv) watery diarrhea among infants which is associated with enteropathogenic *E. coli* (Mamun *et al*., 1993; Sang *et al*., 2012).

All the six pathotypes of *E. coli; EPEC*, ETEC, EIEC, EHEC, EAEC and DAEC, have been linked to diarrheal illness in various areas of Africa amongst children under five years (Sang *et al*., 2012), HIV-positive individuals (Okeke, 2009) and visitors from abroad (Zhi-Dong *et al*., 2002). Every pathotype has distinct disease causing mechanism to cause diarrhea. They have also been isolated from different parts of Africa. However, the true magnitude of these pathogens remains unspecified since limited molecular epidemiological studies have been performed on these organisms (Okeke, 2009).

Enteric pathogens resistant to current antimicrobial agents have increased worldwide due to the widespread and uncontrolled use of antimicrobials. *E. coli* isolates from a study conducted in four provinces in Kenya, showed resistance to one or more antimicrobials including (i) gentamicin, (ii) ampicillin, (iii) chloramphenicol, (iv) tetracycline and (v) trimethoprim/sulphamethoxazole (Sang *et al*., 2012). However, Sang *et al.* (2012) among the Maasai community of Kenya reported low levels of antibiotic resistance compared to other studies in Kenya. This might be due to low exposure and uptake of antibiotics in the community which have been known to heavily rely on traditional medication. Consequently antibiotics which have established resistant in other areas of Kenya were found to be effective among this community (Sang *et al.*, 2012).

1.2 Problem Statement

Among the under five years, DEC pathotypes namely (i) ETEC, (ii) EPEC and (iii) EAEC are enteric pathogens of great importance and are attributed to 30 to 40% of all the diarrheal episodes in low income countries (Clarke *et al*., 2001; O'Ryan *et al.*, 2005; WHO, 2013) where childhood diarrhea is the second leading cause of death amongst children less than five years (about 1.5 million annually) (Black *et al*., 2008; UNICEF/WHO, 2009). Enterotoxigenic *E. coli* is the commonest bacterial pathogen

associated with endemic forms of childhood diarrhea (Dutta *et al*., 2014; Sang *et al*., 2012) and travellers' diarrhea (Zhi-Dong *et al*., 2002).

Emerging strains of diarrheagenic *E. coli* (i.e. (i) *E. coli* O157, (ii) Pathogenic *E. coli* species and (iii) EAggEC) are responsible for severe diarrhea outbreaks in Africa (Bundi *et al*., 2013; Effler *et al*., 2001). Recently in Kenya, Bundi *et al*. (2013) isolated EAEC which was associated with the 2009 diarrheal disease outbreak in Mandela District in Kenya where majority of cases were paediatric. Additionally, Shiga-toxin producing *E. coli*, as high as 24.1%, was isolated among the Maasai population of Kenya with bloody diarrhea causing Intimin-positive STEC strains affecting mostly children below 5 years, while Intimin-negative STEC stains dominating in adults (Sang *et al*., 2012).

On the other hand multiple reports of antimicrobial resistance among diarrheagenic *E. coli* pathotypes have been documented in Kenya (Bii *et al*., 2005; Brooks *et al*., 2006; Sang *et al*., 1997; Sang *et al*., 2012). This distribution of diarrheagenic *E. coli* strains, the emergence of new virulent enteric pathogens and emerging antibiotic resistance threatens the effectiveness of successful treatment of diarrheal infections posing a public health concern locally, nationally and globally which if not investigated and intervention initiated, could result to increased morbidity, disease burden and mortality. Antibiotic resistance problem has been and remains neglected globally, in part owing to lack of documentation through a systematic surveillance system. In addition, the increasing interconnections between countries and the globalization of trade and travel has further contributed to the risk of importing bacteria or genes that jeopardize effective treatment or the prevention of bacterial infections (Amabile-Cuevas, 2010).

1.3 Justification

Baseline data from the rapid assessment of antimicrobial resistance in selected (Mbagathi District Hospital, Thika and Machakos level 5 hospitals, Nakuru and Nyeri Provincial Hospitals, Kenyatta National Hospital and National Public Health Laboratories Services Microbiology Department) public health and clinical laboratories done in March 2013 reported *E. coli* as the commonest isolated enteric *(*12.4 %) from stool culture among both children and adults. Thika Level 5 Hospital laboratory reported the highest *E. coli* isolates from the microbiology register review (January – December 2012) (Odhiambo *et al*., 2014). No further characterization was done to these isolates due to limited capacity of the laboratory.

Pathogenic species of *E. coli* can neither be differentiated from other stains nor from one another by (i) morphological presentation on culture media nor (ii) their biochemical characteristics. To determine whether the isolated *E. coli* isolates are pathogenic or merely a constituent of the normal flora, several methods can be performed to further characterize the strains and document the proportion of diarrhea which is associated with the different diarrheagenic *E. coli* pathotypes. Molecular methods have been documented to be highly specific and rapid mode of diagnosis. This study informed us on the prevalence of diarrheagenic *E. coli* and their AST profile among patients seeking diarrhea treatment at Thika level 5 Hospital. This informed better management of diarrheal illness.

1.4 Research Question(s)

1) What is the prevalence of diarrheagenic *E. coli* in faecal specimens among patients with diarrhea in Thika level 5 Hospital from April to July 2014?

2) What are the circulating pathotypes of diarrheagenic *E. coli* among patients with diarrhea in Thika Level 5 Hospital from April to July 2014?

3) What are the antimicrobial susceptibility patterns of diarrheagenic *E. coli* among patients with diarrhea in Thika Level 5 Hospital from April to July 2014?

1.5 Objectives

1.5.1 Broad objective

To isolate, identify and characterize diarrheagenic *E. coli* among diarrheal patients in Thika Level 5 Hospital and determine their antimicrobial susceptibility pattern.

1.5.2 Specific objective

1) To determine the prevalence of diarrheagenic *E. coli* in faecal specimens among patients with diarrhea in Thika level 5 Hospital from April to July 2014.

2) To determine and characterize the pathotypes of circulating diarrheagenic *E. coli* among patients with diarrhea in Thika Level 5 Hospital from April to July 2014.

3) To determine antimicrobial resistant profiles of the *E. coli* isolates among patients with diarrhea in Thika Level 5 Hospital from April to July 2014.

CHAPTER TWO

LITERATURE REVIEW

Diarrhea is disease of public health importance globally, particularly in countries with limited resources where sanitation facilities are inadequate (Okeke, 2009). Diarrheagenic *E. coli* (DEC) is transmitted through consumption of contaminated water or food. Six *E. coli* pathotypes namely (i) EPEC, (ii) ETEC, (iii) EIEC, (iv) EHEC, (v) EAEC and (vi) DAEC are associated with diarrhea and are thus collectively referred to as DEC. This chapter outlines the current knowledge and understanding of DEC pathotypes.

2.1 Enterotoxigenic *Escherichia coli*

Enterotoxigenic *E. coli* (ETEC) attaches on the small intestine using fimbrial adhesins (projections from the bacterial cell surface). They produces two toxins; (i) heat labile enterotoxin (LT) which has similar properties as cholera toxin structurally and functionally and (ii) heat stable enterotoxin (ST) which stimulates production of fluid and electrolytes into the intestinal lumen. These pathotype do not spread to invade epithelial cells of the intestines or other parts but are restricted in the intestinal lumen.

They have been documented as the leading diarrhea causing bacteria among children in low income countries (Okeke, 2009; Gonzales *et al*., 2013), in addition to being the predominant cause of traveler's diarrhea among travellers touring developing tropical countries (Black, 1990). This agrees with a study conducted among the Europeans visiting Mombasa, Kenya which documented an attack rate of 35% associated with ETEC infection (Shaheen *et al*., 2003; Zhi-Dong *et al*., 2002). This diarrhea illness has (i) an abrupt onset, (ii) a short incubation period (14 to 50 hours) and (ii) present as watery, mostly devoid of blood, mucus, or pus. In addition, fever and vomiting might be present in some patients. Enterotoxigenic *E. coli* diarrhea may be (i) mild, brief, and self-limiting or (ii) result in profuse diarrhea as in cholera.

The recommended management of ETEC diarrhea entails maintenance of normal hydration through oral rehydration therapy which is life-saving especially among children below five years (Nataro & Kaper, 1998). Molecular identification of *elt* (heat labile enterotoxin) and *esth* (heat stable enterotoxin) genes defines an ETEC isolate (Nataro & Kaper, 1998; Pass *et al.*, 2000).

2.2 Enteropathogenic *Escherichia coli*

Enteropathogenic *E. coli* (EPEC) produces characteristic attaching and effacing lesions on the intestinal mucosa. They are distinguished by their ability to attach to cultured human epithelial cells *in vitro* exhibiting a pattern known as localized adherence (LA), in which microcolonies form on the surfaces of the cells (Scaletsky *et al*., 1984). An adhesin called intimin is used by the bacteria to attach to the cells of the intestines. Enteropathogenic *E. coli* has locus for enterocyte effacement (LEE) which is a chromosomal pathogenicity island that confers a distinctive ―attaching and effacing phenotype although they do not carry genes for the phage-borne Shiga-toxins of EHEC. Typical EPEC pathotypes also carry a virulence plasmid, which bears genes encoding bundle-forming pili, the plasmid encoded regulator and other putative virulence genes (Nataro & Kaper, 1998). The attachment of the bacteria to the wall of the intestine results into rearrangement of actin in the host cell, leading to significant distortion. Enteropathogenic *E. coli* strains moderately invade host intestinal epithelial cells causing inflammation and immune response. Ultra-structural changes of the mucosal cells of the intestine caused by bacterial "attachment and effacement" are linked to diarrhea disease development among EPEC infected persons. This pathotype has been attributed to diarrheal disease among children in low income countries (Moyo *et al*., 2007). In molecular analysis, EPEC identification is through identification of *eae* Intimin *(*LEE-encoded adhesin) which is a marker for LEE pathogenicity island or *bfpA* (structural subunit of the "bundle-forming pilus" (BFP) genes (Beutin *et al.*, 2005; Nataro & Kaper, 1998; Pass *et al.*, 2000).

Between 1966 through 1989, EPEC O111 was prevalent and has been reported in outbreaks with high fatality rates in Africa (Agbodaze *et al.,* 1988; Agbonlahor *et al*., 1982; Mutanda *et al*., 1987; Senerwa *et al*., 1989; Thoren, 1980; Tobe *et al.,* 1999; Voros *et al*., 1978). During the early and mid-1900s, O111 EPEC was also recovered in outbreaks affecting nursery school in the United States (Tobe *et al.*, 1999; Zhou *et al.,* 2003). However, in the recent years, EPEC has become a less predominant cause of childhood diarrhea in Africa and this is attributed to the 0-6 month exclusive breastfeeding campaign (Cravioto *et al.,* 1991; UNICEF/ WHO, 2009).

2.3 Enteroinvasive *Escherichia coli*

Enteroinvasive *E. coli* (EIEC) just like *Shigella,* invades intestinal epithelial cells. It is characterized by the presence of a large invasive plasmid which encodes (i) the Mxi-Spa type III secretion system and (ii) invasion plasmid antigen (Ipa) effectors, enabling eukaryotic cell invasion and (iii) IcsA, which facilitates bacterial movement from one cell to another *in vivo,* evading the immune system. Other virulent factors include shiga-toxin and pathoadaptive deletions in house-keeping genes (Nataro & Kaper, 1998).

Enteroinvasive *E. coli* infection causes symptoms similar to shigellosis among adults and children with profuse diarrhea and high fever. However limited studies have been directed on it compared to other *E. coli* pathotypes. It is mostly described in outbreaks (Nataro & Kaper, 1998). Molecular identification of EIEC has largely been through demonstrating that the organism possesses *inv* (Invasion plasmid) (Nataro & Kaper, 1998; Pass *et al.,* 2000).

2.4 Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *E. coli* (EHEC) are defined by attaching-and effacing-(A/E) lesions and shiga-like toxin or verotoxins. They use bacterial fimbriae to attach to the intestinal epithelial cells (*E. coli* common pilus, ECP), invade intestinal epithelial cells moderately and carry a shiga toxin encoded phage which causes intense inflammatory response. They are zoonotic food-borne agents associated with diarrhoea outbreaks globally and pose significant public health concerns. It is also called Verocytoxin producing *E. coli* (VTEC) as well as Shiga toxin producing *E. coli* (STEC), due to their toxins similarity to that produced by *Shigella dysenteriae* (Sang *et al*., 2012). Enterohemorrhagic *E. coli* serotype O157:H7 is the most significant EHEC serotype drawing numerous public health interests; however, other serotypes have been isolated in sporadic cases and outbreak. It is responsible for outbreaks where cases presents with (i) abdominal cramps, (ii) bloody diarrheal, and (iii) the life-threatening complications; haemolytic uremic syndrome and sudden kidney failure (Loirat, *et al.,* 2011; Goldwater & Bettelheim, 2012; Sang *et al*., 2012).

In Kenya, it has been reported to cause bloody diarrhea in both children and adults among the Maasai community (Sang *et al.,* 2012). Most molecular assays to demonstrate EHEC are tailored towards identification of genes encoding *stx* (shiga toxin 1and 2) since the demonstration of these genes in a clinical specimen is significant. Other genes sought include *eae* (Intimin) and *hly* (Enterohemolysin) (Nataro & Kaper, 1998; Pass *et al.,* 2000).

2.5 Enteroaggregative *Escherichia coli*

Enteroaggregative *E. coli* (EAEC) are considered as emerging pathogens. They are defined by their characteristic adhering pattern on human epithelial cells culture, that is, aggregative adherence (AA) pattern. This pattern presents as stacked brick-like arrangement on the surfaces of the cells, on the glass or plastic containers (Nataro & Kaper, 1998; Loirat *et al.,* 2011). This property is attributed to the presence of aggregative adherence fimbriae (AAF/I), AAF/II and AAF/ III) whose expression is

positively controlled by the *aggR* gene, located on a large plasmid termed pAA (Nataro *et al*., 2006). Aggregative adherence fimbriae play a central role in EAEC pathogenesis. They facilitate bacterial attachment to the epithelial cells of the intestine membrane and the formation of a thick biofilm within the mucus layer covering the epithelium. This allows the bacteria to continuously colonize the intestinal mucosal membrane causing disease. Aggregative adherence fimbriae-mediated adherence causes inflammatory responses, including secretion of pro-inflammatory cytokines and recruitment and infiltration of neutrophils.

Illness is the outcome of the interaction between bacteria and infected person; from the bacterial attachment to the intestinal epithelial cells, distortion of the mucosal membrane and a finally inflammatory response of the intestinal mucosa (Nataro *et al*., 2006). Enteroaggregative *E. coli* pathogenesis is also facilitated by "dispersin", a surface coat required for proper dispersal of aggregative adherence fimbria (AAF) on the bacterial surface (Sheikh *et al.,* 2002). Dispersin is also a possible immunogen to prevent EAEC infections. The gene implicated for formation of "dispersin" is called *aap* (dispersin secretory protein) gene, formally known as *aspU* (EAEC secreted protein U) (Czeczulin *et al.,* 1999).

Virulence genes sought for identification of EAEC includes (i) *PAA* (aggregative adherence plasmid), (ii) *aggA* (aggregative adherence fimbriae 1), (iii) *aafA* (aggregative adherence fimbriae 2), (iv) *set* (shigella enterotoxin 1), (v) *sen* (shigella enterotoxin 2), (vi) *aggR* (transcriptional activator), (vii) aat secretion CVD432 (anti-aggregation protein transporter), (viii) *aap* (dispersin secretory protein) or (ix) *aspU* (EAEC secreted protein U) (Aslani *et al.,* 2011; Czeczulin *et al.,* 1999; Villaseca *et al.,* 2005). Enteroaggregative *E. coli* infection is linked to persistent watery diarrhea among children in low income countries (Nataro & Kaper, 1998). Enteroaggregative *E. coli* is also attributed to diarrheal disease among travellers and HIV positive persons (Huang *et al*., 2006). Moreover, EAEC has been isolated in outbreaks (Bundi *et al*., 2013) and non-outbreak settings in low and high income countries (Kaur *et al*., 2010).

2.6 Diffusely adherent *Escherichia coli*

Diffusely adherent *Escherichia coli* (DAEC) pathotypes are defined by their characteristic attachment pattern exhibited by the bacteria on human epithelial cells culture known as diffuse adherence (DA). The pattern presents the bacteria uniformly covering the entire cell surface (Scaletsky *et al*., 1984). Two putative adherence factors have been described for DAEC strains; (i) surface fimbriae designated F1845 (Bilge *et al.,* 1989) and (ii) an outer membrane protein (adhesin) designated AIDA-I (Benz & Schmidt, 1989; Nowicki *et al.,* 1990; Croxen & Finlay, 2010). This pathotype is attributed to diarrheal disease in various locations (Baqui *et al*., 1992; Giro´n *et al.,* 1999; Gunzburg *et al*., 1993; Jallat *et al*., 1993; Croxen & Finlay, 2010). Scaletsky *et al*. (2002) confirmed the association of DAEC with age-dependent diarrhea, under 1 year children. In molecular DNA analysis, *daaC* (AIDA-I) probe has been used for identification of DAEC (Isabel *et al.,* 2002).

2.7 Diarrhea Treatment

Infectious diseases control is highly threatened by the rising number of antimicrobial resistance micro-organisms. This has led to longer hospital stay, prolonged illness, high cost of treatment, treatment failure and greater risk of death (Laxminarayan, 2003; Gaude & Hattiholi, 2013). The main cause of antimicrobial resistance is bacterial genetic mutation which is led by prolonged used of antimicrobials, non-adherence to recommended doses, irrational use of antimicrobials in our food chains (animal husbandry and agriculture), mobile transfer of resistant genes and lack of surveillance (Linton, 1986; Amabile-Cuevas, 2010; Baidouri *et al.*, 2014; Landecker, 2015; Langerndorf *et al.,* 2015).

In Kenya, diarrhea case management policy recommends that diarrhea among children under-fives years of age should be treated with Oral Rehydration Salts (ORS), Zinc tables and Vitamin A; if they have not received the vitamin in the last one month. Antimicrobial are used in suspected or proven dysentery or Cholera. Erythromycin and chloramphenicol are the recommended first and second line drugs of choice in Cholera management in children receptively. On the other hand,

ciprofloxacin should be prescribed in childhood dysentery (Policy Guidelines for the Management of Diarrhea in Children Below Five Years in Kenya, 2014). In adults, antimicrobial agents use is confined to the etiology and or clinical circumstances of an individual. For empirical diarrhea case management in Kenya, the recommended treatment includes tetracycline, ampicillin and trimethoprimsulpha-methoxazole. Commonly prescribe antimicrobials in diarrhea case management include ampicillin, erythromycin, tetracycline, chloramphenicol, amoxicillin/clavulanic acid, cefazolin, cefuroxime, cefuroxim, cefoxitin, cefotaxime, ceftazidime, ceftriaxone, cefepime, meropenem, amikacin, gentamicin, ciprofloxacin, fosfomycin and trimethoprim /sulfamethoxazole. Due to inadequate laboratory facilities, many clinicians have been prescribing the medications without laboratory confirmation (Odhiambo *et al.,* 2014).

Many of the studies that have been performed looked for specific but not all DEC pathotypes, or did not focus on identifying pathogenic pathotypes or were responding to an outbreak situation. This study explored the different diarrheagenic *E. coli* pathotypes among patients who sought treatment from April to July 2014 at Thika Level 5 Hospital and antimicrobial susceptibility pattern of the pathotypes isolated.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

A prospective cross-sectional study was carried out among patients of all ages seeking diarrhea treatment in Thika Level 5 Hospital to establish the prevalence, types of circulating diarrheagenic *E. coli* strains and their antimicrobial susceptibility profile from April to July 2014.

3.2 Study Site

Thika Level 5 Hospital is public hospital in Thika Sub-County located approximately 50 km north east of Nairobi, in Kiambu County, Kenya. This hospital reported the highest *E. coli* organism isolation from patients with diarrhea during the rapid assessment of antimicrobial resistance in Nairobi and adjacent districts in March 2013 (Odhiambo *et al.,* 2014). The *E. coli* isolates were not further characterized due to lack of molecular diagnosis capacity leading to non-evidence based diarrhea case management. This creates a room for improper diarrhea case management and emergence of drug resistance.

3.3 Study Population

The study population involved patients of all ages seeking diarrhea treatment at Thika Level 5 Hospital from April to July 2014 who had not taken antibiotics within 72 hours of diarrhea onset. Diarrhoea case definition was having at least three loose stools within 24 hours, or any number of watery stools. All diarrhea patients meeting the case definition were enrolled until the sample size was attained.

3.3.1 Inclusion Criteria

Study participants were consenting and assenting diarrhoea patients of all ages from both in and out patient departments seeking treatment for diarrhoea at the hospital.

3.3.2 Exclusion Criteria

Diarrhea patients who had taken antimicrobials within 72 hours of symptoms onset and those unwilling to be involved in the research.

3.4 Sample Size Determination

To calculate the sample size, we used a prevalence of 50% estimated by Fisher and Van Belle (2004) as no previous similar study had been done at Thika Level 5 Hospital.

$$\mathbf{n= \frac{t^2 \times p(1-p)}{m^2}}$$

Where: n= required sample size

t = Confidence Interval for 95% CI is 1.96

p = prevalence of *E. coli,* at 50%

q = (1-p) and m = precision (margin of error at 5%), i.e. 0.05

$$n= \frac{(1.96)^2 \times 0.5 \times 0.5}{(0.05)^2}$$

n= 384 participants

3.5 Sampling Procedure

All consenting or assenting patients (caregivers, parents or guardians) seeking diarrhea treatment at Thika level 5 Hospital during the period of the study were recruited until the sample size was achieved.

3.6 Recruitment Procedure

3.6.1 Out-patient

Study participants were recruited on voluntary basis on reporting to the hospital laboratory. Before enrollment, the researcher informed the prospective participants about the objective of the study, potential risks and benefits of participation. They were later asked to give a written informed consent or assent by signing the consent/ assent form after all study information had been provided in a language they understood (Appendix IV and VI). Study participants were those who consented or assented. The participants were then assigned a participant identification number (PIN) to ensured confidentiality is maintained among the study participants. The researcher then administered a structured questionnaire (Appendix III). Each questionnaire was labeled with the participant's PIN.

3.6.2 In-patient

Each ward had a pre-trained study nurse who identified study participants based on the inclusion and exclusion criteria. The study nurse gave detailed information with regard to this study including potential risks and benefits of participation to potential participants. The study participants were those who gave a written consent or assent after all study information had been provided. The study nurse then assigned a participant identification number (PIN) to each person who consent or assent to participate to ensure confidentiality. The nurse proceeded to administer the study questionnaire (Appendix III) labelled with the participant's PIN.

3.7 Questionnaire

Information on demographics, date of symptoms onset, water hygiene and sanitation practices were obtained using a structured questionnaire (Appendix III).

3.8 Variables

Variables were abstracted from the laboratory request form and study questionnaire. All information, except study participants' name was transferred from the request form to the research register for data archiving. They included independent variables; (i) EPEC, (ii) ETEC, (iii) EIEC, (iv) EHEC and (v) EAEC, and dependent variables; (i) age, (ii) date of diarrhea on set, (iii) sign and symptoms, (iv) source of drinking water, (v) water storage container, (vi) size of the month of water storage container and (vii) toilet structure.

3.9 Sample Collection

Stool samples were collected on patient presentation at the laboratory or recruitment from the in-patient department. Stool samples were collected in a plastic sterile stool container following instructions from the research team. Children samples were collected from their diapers and placed in the stool collecting container by the parents / caregivers following study team instructions.

3.10 Laboratory Analysis

3.10.1 Stool Wet Microscopy

A stool wet mount was prepared by picking a small amount of the stool, placing it on a microscope slide, emulsifying it with 0.2 ml of normal saline, covering it with a cover slide and observing it microscopically at magnification x10. This was used to identify protozoan trophozoites, cysts and helminth eggs and larvae.

3.10.2 Stool culture and identification of enteric organisms

Shigella, *Salmonella*, *Vibrio cholerae* and *E. coli* isolates were sought since they are the main cause of inflammatory diarrhea in low income countries (Barletta *et al*., 2013). All stool samples were plated onto (i) MacConkey, (ii) Xylose-Lysine-deoxycholate (XLD) media and (iii) Thiosulfate Citrate Bile salts Sucrose (TCBS) media and incubated at 37^0C for 18 – 24 hours. Prior to sample plating on XLD, the stool specimen was inoculated in Selenite F Broth for enrichment purposes

aerobically at 37^0C for 18 – 24 hours. *Salmonella* colonies appeared as red colonies (Non Lactose Fermenters – with black centers) in XLD media while *E. coli* colonies fermented lactose in MacConkey media to yield pink colonies.

3.10.3 Biochemical screening tests

After overnight growth, five to ten single colonies with typical *E. coli* morphology were selected and characterized on the basis of their biochemical reactions guided by key biochemical properties as illustrated in the laboratory analysis flow chart (Appendix X). The Indole tubes were inoculated by stubbing straight into the medium by using a sterile straight wire (CDC/WHO, 1999).

3.10.4 Identification of *E. coli* isolates by VITEK 2 Compact

VITEK 2 Compact is an automated microbiology analyzer which offers accurate, rapid and standardized identification and antimicrobial susceptibility testing. Three colonies of lactose-fermenting colonies identified as *E. coli* by their colonial morphology and biochemical properties were further tested using the VITEK© 2 Compact; Identification Gram-Negative Bacilli (ID-GNB) cards (bio-Me´rieux, Marcy L'Etoile, France) (Pincus, 2005). The identification (ID) suspension was prepared by emulsifying these colonies into 3ml of 0.45% NaCl sterile saline (pH= 4.5 – 7.0) into a 12x75mm clear plastic tube. The optical density of the solution was checked with DensiCHEK Plus to 0.5 McFarland before placing ID-GNB card and the saline tube into the cassette for filling and loading into the VITEK 2 Compact for identification of *E. coli.*

3.10.5 Extraction of *E. coli* DNA by Qiacube®

This procedure is adapted from Qiagen DNeasy DNA extraction protocol for bacterial cultures (QiaDNA, 2006) and was performed using Qiacube® automated nucleic acid extraction system. The spin procedures are conducted in columns-collection tubes to prevent contamination from one sample to another and also from the samples to the operators.

1) 2 ml tubes were labeled with specimen identification numbers.
2) Overnight grown bacterial colonies were picked using inoculating loops, suspended in 180 ul of ATL buffer and vortexed 10-20s.
3) 25 ul of proteinase K was added to the tubes. The tubes were then vortexed.
4) 200 ul of Buffer AL were added to the tubes. The tubes were then vortexed.
5) The tubes were incubated at 56° C for 30 minutes after which 200 ul of 100% ethanol was added to the tubes and vortexed.
6) The tubes content were transferred to labeled spin columns and centrifuged at 10,000 x g for 1 minute.
7) The collection tubes were changed, old columns discarded with the filtrate and new collection tubes added to the columns.
8) The columns were washed with 500 ul of buffer AW1 (contain guanidine hydrochloride) and the content centrifuged at 10,000 x g for 1 minute.
9) The collection tubes were changed, old columns discarded with the filtrate and the columns placed in new collection tubes.
10) The columns were washed with 500 ul of buffer AW2 and the content centrifuged at 20,000 x g for 3 minutes.
11) The columns were then transferred to 1.5 ml tubes and 200 ul of buffer AE was added. The tubes were then left to stand at room temperature for 5 minutes. This yields more DNA compared to letting the tube stand for 1 minute.

12) Tubes were then centrifuged at 10,000 x g for 1 minute, columns discarded and the harvested DNA stored at 4^0C.

3.10.6 Identification of Diarrheagenic *E. coli* by polymerase chain reaction

After *E. coli* DNA extraction, the extracts were subjected to multiplex Real Time Polymerase Chain Reaction (RT-PCR) for detection of virulence genes. The PCR targets were: ETEC *elt* (heat labile enterotoxins) and *est* (heat stable enterotoxin), EHEC *stx* (Shiga toxins), EAEC *aggR* (activator aggregative adherence regulator) and *aspU (EAEC*-secreated protein U gene), EIEC *ipaH* (invasion plasmid antigen) and EPEC *eae* (intimin). The reaction was carried out in 0.2 ml thin-walled PCR tubes using a 25 ul reaction mixture containing puReTaq™ Ready-To-Go™ Polymerase Chain Reaction (PCR) Beads (GE Healthcare, Buckinghamshire, HP7 9NA UK), 20 ul of double distilled water, 2 ul of extracted *E. coli* DNA templates and 3 ul of primers mixer (Table 3.1) in each reaction tube.

Table 3.1: Diarrhoeagenic *E. coli* Multiplex PCR primers sequences

	Sequence (5' to 3')	Target Gene	Amplicon size (bp)	Reference
EPEC	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	*eae*	881	Oswald *et al.,* 2000
EHEC	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT	*stx*	518	Yamasaki *et al.,* 1996
ETEC	TTAATAGCACCCGGTACAAGCAGG CCTGACTCTTCAAAAGAGAAAATTAC	*est*	147	Hornes *et al.,* 1991
ETEC	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	*elt*	322	Tamanai & Jolivet et *al.,* 1994
EIEC	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	*ipaH*	*619*	Sethabutr *et al.,* 1993
EAEC	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	*aggR*	254	Ratchtrachenchai *et al.,* 1997
EAEC	GCCTTTGCGGGTGGTAGCGG AACCCATTCGGTTAGAGCAC	*aspU*	282	Claudia *et al.,* 2003

Positive controls containing targeted virulence genes; EAEC strain 17-2, ETEC ATCC 35401 (*eltB*, *estA*), EHEC ATCC 43890 (*vt1*, *eaeA*), EHEC ATCC 43889 (*vt2*, *eaeA*), EPEC ATCC 43887 (*eaeA*, *bfpA*), EIEC ATCC 43893 (*ial*) and negative control without virulence genes were used in every amplification round. PCR cycle parameters were; initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 54°C for 45 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The PCR products were electrophoresed on 2% L03

agarose (TaKaRa Bio Inc. Shiga, Japan) with ethidium bromide and visualized on an ultraviolet trans-illuminator, the Gel Doc™ EZ Imager (BioRad LaboratoriesMarne-la-Coquette,France).

3.10.7 Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns for diarrheagenic *E. coli* were determined using VITEK 2 compact, Antimicrobial Susceptibility Testing (AST) card for Gram-negative bacilli, automated microbiology analyzer whose breaking point are guided by Clinical and Laboratory Standards Institute guidelines with *E. coli* ATCC 35218 strain being used as the test standard. The AST suspension was processed by transferring 145 microliters of the identification suspension into 3ml of 0.45% NaCl sterile saline (pH= 4.5 – 7.0) into a 12x75mm clear plastic tube. The AST card and the saline tube were then placed into the cassette for filling and loading into the VITEK© 2 Compact (bio-Me´rieux, Marcy L'Etoile, France) for examination. The following antibacterial agents: (i) ampicillin, (ii) amoxicillin/clavulanic acid, (iii) ampicillin/sulbactam, (iv) piperacillin/tazobactam, (v) cefazolin, (vi) cefuroxime, (vii) cefuroxime axetil, (viii) cefoxitin, (ix) cefotaxime, (x) ceftazidime, (xi) ceftriaxone, (xii) cefepime, (xiii) aztreonam, (xiv) meropenem, (xv) amikacin, (xvi) gentamicin, (xvii) ciprofloxacin, (xviii) nitrofurantoin and (xix)trimethoprim /sulfamethoxazole were used (Appendix IX). The antibacterial agents were based on the VITEK antimicrobial susceptibility card which is CLSI guided.

3.11 Data Management and Analysis

3.11.1 Data collection

Data was obtained from completed structured questionnaires following face to face interviews and stool sample results after laboratory analysis. The questionnaires (Appendix III) were administered by the research team (trained study nurses, study laboratory officer and the researcher).

3.11.2 Data storage and analysis

All questionnaires were coded and validated on daily basis during data collection period. Data was entered into a computer and saved in Excel workbook after quality cleaning. Data analysis was done using Epi info version 3.5.1 (CDC Atlanta USA). Descriptive analysis of frequencies and proportion was determined for both the dependent and independent variables. The association between independent variables and level of education, source of drinking water, water storage container, size of the month of water storage container and toilet structure were determined using odds ratio, chi-square and Fisher exact test. Any variable with $p<0.05$ was considered statistically significant. All factors statistically significant in bivariate analysis were put in conditional logistic regression model with stepwise backward elimination, to come up with the independent factor associated with DEC infection.

3.12 Risks and Benefits to the Client

There was no risk involved in stool specimen collection. The participant benefited from results of stool culture, sensitivity diagnosis test as well as the PCR results.

3.13 Data and Information Protection

Participants were assigned a participant identification number (PIN). This ensured anonymity for the study participants. Each questionnaire was labeled with the participant's PIN and not personal identifiers (e.g. Name and identification number). Access to the computer used for storage of study data was limited to the researcher and limited authorized persons. The computer and study data was protected by means of a password.

3.14 Approval and Ethical Clearance

The study protocol was approved by Kenyatta National Hospital research committee (Appendix I) and Thika Level 5 Hospital research board (Appendix II) before the

commencement of the study. The participants' data obtained from the patient laboratory request forms were coded, stored properly and accessed by only authorized persons to avoid a breach in confidentiality.

3.15 Dissemination of Data

Data generated was reported in study review meetings with the hospital administration, presented in scientific conferences (Appendix XIII) and meetings and published in African Journal of Health Sciences (Appendix XI and XII). Identifying data was no used in any presentation and publication. Confidentiality of subjects was maintained.

CHAPTER FOUR

RESULTS

4.1 Socio-demographic and clinical characteristics of study participants

Between April 2014 to July 2014, 402 participants were enrolled; 222 (55%) female with a median age of 14 (IQR: 3-31) years (Table 4.1).

Table 4.1: Socio-demographic characteristics of the study participants

Variable	Participants	N*= 402 (%)
Age	< 5 years	133 (33)
	> 5 years	269 (67)
Gender	Male	180 (45)
	Female	222 (55)
Level of education	No formal education	6 (2)
	Primary	114 (28)
	Secondary	204 (51)
	Post-secondary	78 (19)
Source of drinking water	Piped to house	243 (61)
	Piped (common point) outside house	74 (18)
	Borehole	73 (18)
	Rivers/springs	12 (3)
Water storage container	Jerry can	246 (61)
	Bucket	96 (24)
	Tank	30 (7)
	Drum	30 (7)
Boil drinking water	No	246 (64)
	Yes	156 (36)
Treat drinking water	No	359 (89)
	Yes	43 (11)
Toilet description	Serving a plot (communal) **	270 (67)
	Built into the house (Single family)	114 (28)
	Pit latrine	18 (5)

N* = number of participants enrolled, ** multiple families in a residential setting

Two hundred and sixty seven (67%) samples were from persons above-five years of age. Where diarrhea patients were under 5 years, education, water, hygiene and sanitation information was sought from the care giver or parent.

4.1.1 Clinical presentation of the participants

Of the 402 participants, 67% presented with abdominal pain and 55% with fever (Figure 4.1). There were variations in the interval between the onset of diarrhoea symptoms and collection of stool specimens; 270 (67.2%) participants presented within 1-3 days, 90 (22%) presented within 4-6 days and 42 (10%) presented after 6 days. *Entamoeba histolytica* was identified in 42 (10%) diarrhoea patients.

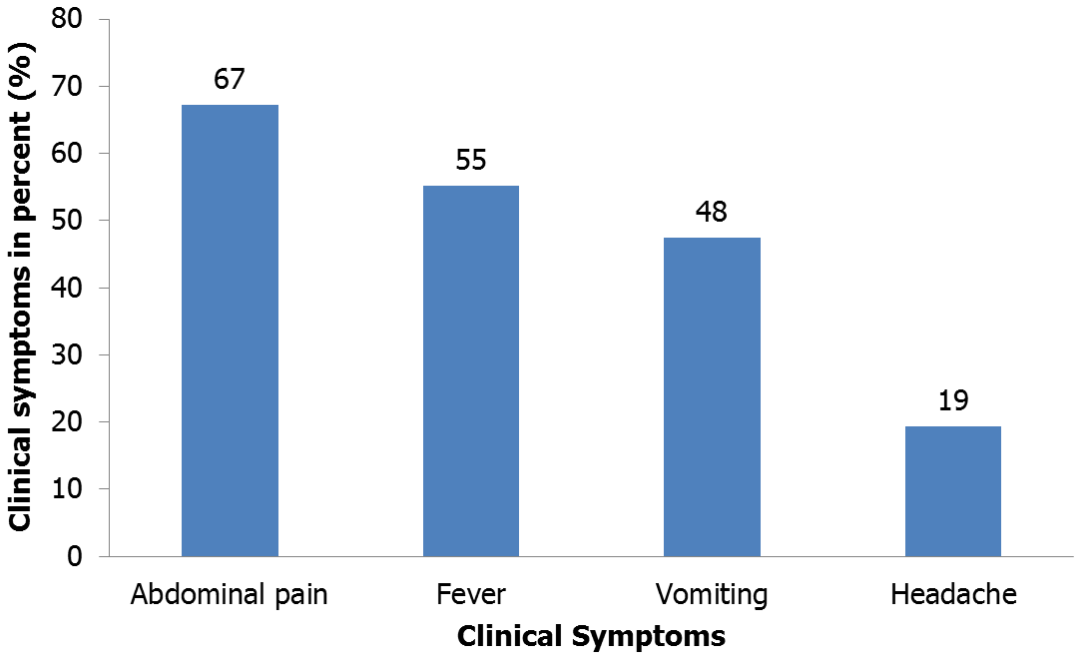

Figure 4.1: Clinical presentation of diarrhoea patients in Thika Level 5 Hospital, April to July 2014

4.2 Characterization of Diarrheagenic *E. coli* isolates

Of the total 402 stool cultures performed, 101 (25%) obtained no pathogenic growth. Of the 301 cultures with pathogenic growth, 269 (89%) *E. coli* were isolated from both children and adult samples. Non-enteric pathogens observed included *Citrobacter spp*., *Klebsiella spp.* and *Pseudomonas spp*. (Table 4.2).

Table 4.2: Bacterial pathogen isolated among diarrhoea patients in Thika Level 5 Hospital, April to July 2014

Organisms isolated	N*= 301 (%)
Escherichia coli	269 (89.4)
Pseudomonas spp	13 (4.3)
Citrobacter spp	12 (4.0)
Klebsiella spp	6 (2.0)
Salmonella spp	1 (0.3)

Legend: N*= number of stool samples with pathogenic isolates

All *Escherichia coli* isolates exhibited a positive reaction on indole. These isolates were also examined using VIKET 2 Compact with 100% positivity for *E. coli*. This was followed by PCR to characterize diarrheagenic *E. coli*. A total of 72 (27%) diarrheagenic *E. coli* were identified comprising 60 (83.3%) EAEC, 6 (8.3%) EPEC and 6 (8.3%) ETEC pathotypes (Table 4.3). Thus the period prevalence of diarrheagenic *E. coli* was 17.9% (72).

Table 4.3: Distribution of Diarrhoeagenic *E. coli* isolates among diarrhoea patients in Thika Level 5 Hospital, April to July 2014

Age in years	EAEC n* (%)	EPEC n* (%)	ETEC n* (%)
0 - 5	31 (52)	0 (0)	0 (0)
6 – 10	0 (0)	0 (0)	6 (100)
11 – 15	0 (0)	0 (0)	0 (0)
16 – 20	1 (2)	6 (100)	0 (0)
21 – 25	0 (0)	0 (0)	0 (0)
26 – 30	12 (20)	0 (0)	0 (0)
31 – 35	10 (17)	0 (0)	0 (0)
36 – 40	0 (0)	0 (0)	0 (0)
41 – 45	6 (10)	0 (0)	0 (0)
Total	**60 (100)**	**6 (100)**	**6 (100)**

Legend: n*= number of diarrhoeagenic *E. coli* isolates

4.2.1 Demographic characteristics of Diarrheagenic *E. coli* infected patients

Of the 72 patients with DEC infection, 42 (58%) were female with a median age of 8 (IQR: 2-28) years, 25 (35%) were under-five years, 54 (75%) had secondary education, 54 (75%) did not boil drinking water, 72 (100%) did not treat water and 48 (67%) shared toilets (Table 4.4). Where diarrhea patients were under-5 years, education, water, hygiene and sanitation information was sought from the care giver or parent.

Table 4.4: Socio-demographic characteristics of patients with Diarrheagenic *E. coli* among diarrhoea patients in Thika Level 5 Hospital, April to July 2014

Variable	Participants	N*= 72 (%)
Age	< 5 years	25 (35)
	> 5 years	47 (65)
Gender	Male	30 (42)
	Female	42 (58)
Level of education	No formal education	0 (0)
	Primary	12 (17)
	Secondary	54 (75)
	Post-secondary	6 (8)
Source of drinking water	Piped to house	39 (54)
	Borehole	19 (26)
	Piped outside the house (at a common point)	14 (19)
Water storage container	Jerry can	48 (67)
	Bucket	18 (25)
	Tank	6 (8)
Boil drinking water	Yes	18 (25)
	No	54 (75)
Treat drinking water	No	72 (100)
Toilet description	Serving a plot	48 (67)
	Built into the house	18 (25)
	Pit latrine	6 (8)

Legend: N*= number of participants with diarrheagenic *E. coli*

The interval between onset of symptoms and collection of specimens was 1-3 days for all patients where ETEC and EPEC were isolated. However, longer duration was presented by patients infected with EAEC; 48 (80%) patients presenting within 1-3 days, 6 (10%) within 4-6 days and 6 (10%) after 6 or more days. *Entamoeba histolytica* was identified in 17% (12) of patients with diarrheagenic *E. coli*.

4.2.2 Bivariate and multivariate analysis of factors associated with Diarrheagenic *E. coli*

Bivariate analysis was performed to determine any possible risk or protective factors for diarrheagenic *E. coli* in the study population. The odds of DEC infection was slightly over one time higher among the under-five years (OR: 1.13, 95% CI: 0.64-2.00), female (OR: 1.23, 95% CI: 0.71-2.12), participants drinking water piped into a common point (OR: 1.08, 95% CI: 0.71-2.12) and those who stored water in buckets (OR: 1.27, 95% CI: 0.67-2.39), jerry can (OR: 1.01, 95% CI: 0.57-1.79) and tanks (OR: 1.03, 95% CI: 0.39-2.74) as well as those who used communal toilets (OR: 1.01, 95% CI: 0.57-1.79) but the association in this study was not significant at the 95% level of confidence.

No significant association with DEC infection was found with age, gender, water storage container and toilet description on the study participants. Drinking borehole (OR: 2.26, 95% CI: 1.16-4.38), un-treated ($P<0.05$) and un-boiled (OR: 2.58, 95% CI: 1.41-4.70) water was associated with DEC infection as well as lack of post-secondary education (OR: 3.45, 95% CI: 1.40 – 8.46) (Table 4.5). After adjusting for age, sex, level of education, type of water storage container and type of toilets, drinking un-boiled water (OR: 2.51, 95% CI: 1.36-4.61) and lack of post-secondary education (OR: 3.32, 95% CI: 1.34-8.22) were independently associated with DEC infection (Table 4.6).

Table 4.5: Bivariate analysis of factors associated with Diarrhoeagenic *E. coli* among diarrhoea patients in Thika Level 5 Hospital, April to July 2014

Socio demographic Characteristics	Odds Ratio (OR)	Confidence Interval (CI)	Two-tailed P-value 95% CI
Education Level			
Postsecondary	Reference		
No postsecondary	3.45	1.40 – 8.46	0.0078
Source of drinking water			
Piped	Reference		
Borehole	2.26	1.16 – 4.38	0.0236
Drinking boiled water			
Yes	Reference		
No	2.58	1.41 – 4.70	0.0028
Drinking treated water			
Yes	Reference		
No	----	----	0.0003

Table 4.6: Multivariate analysis of factors associated with Diarrhoeagenic *E. coli* among diarrhea patients in Thika Level 5 Hospital, April to July, 2014

Variable	AOR*	95% C.I**	Coefficient	S.E***	Z-Statistic	P-Value
Drinking un-boiling water	2.51	1.36-4.61	0.92	0.31	2.953	0.0032
No post-secondary	3.32	1.34-8.22	1.20	0.46	2.593	0.0095

Legend: AOR* = Adjusted Odds Ratio,
95 % C.I** = Confidence Interval
SE = Standard Error

4.3 Antimicrobial susceptibility pattern of Diarrhoeagenic *E. coli*

All the 72 DEC isolates were subjected to antimicrobial susceptibility testing. The isolates were 100% sensitive to ciprofloxacin (quinolone), meropenem (carbapenem), cefoxitin (cephamycin), amikacin and gentamicin (aminoglycosides). High resistance was observed in ampicillin (92%), trimethoprim /sulfamethoxazole (92%) and amoxicillin/clavulanic acid (85%) while low resistance trends were observed among other antimicrobials; piperacillin/tazobactam (7%), cefuroxime, cefuroxime axetil, cefotaxime, ceftazidime, ceftriazone, cefepime and aztreonam (15%), cefazolin (22%) and nitrofurantoin (24%) (Figure 4.2). Sixty six (92%) of the 72 DEC isolates were resistant to two or more antimicrobial agents (multidrug resistant). The predominant antimicrobial resistance combination profile was ampicillin and co-trimoxazole (63%). This was followed by ampicillin- co-trimoxazole-amoxicillin/clavulanic acid and ampicillin/sulbactam both at 14%.

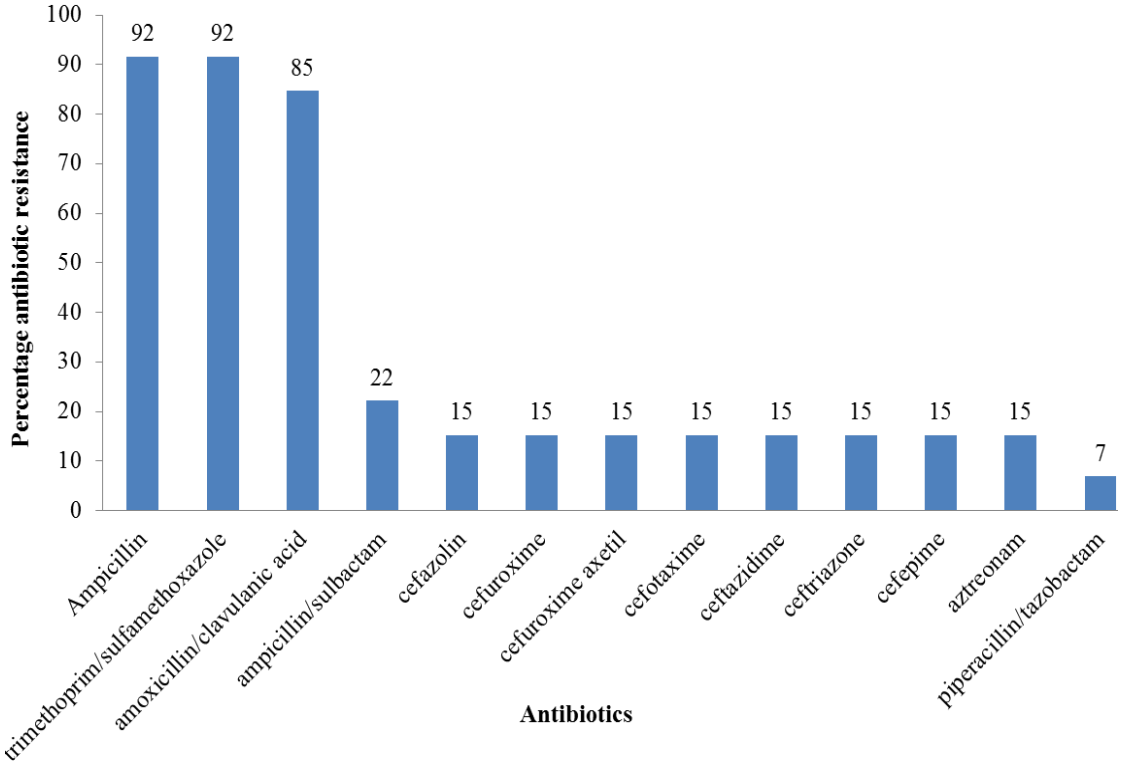

Figure 4.2: Antimicrobial resistance patterns of Diarrheagenic *E. coli* isolates from diarrhoea patients in Thika Level 5 Hospital, April to July 2014

CHAPTER FIVE

DISCUSSION

This study documents DEC infection among patient seeking diarrhoea treatment at Thika Level 5 Hospital between April to July 2014. Three DEC pathotypes were isolated. These pathotypes showed high proportion of resistance to commonly prescribed antimicrobials; ampicillin, trimethoprim /sulfamethoxazole and amoxicillin/clavulanic acid but all were sensitive to two aminoglycosides and one carbapenem, quinolone and cephalosporin.

5.1 Diarrhoeagenic *E. coli* Characterization; Socio-demographic and clinical characteristics of infected patients

All ETEC and EPEC infected persons were over-five years of age in our study. This is inconsistent with other studies where ETEC has been largely associated with childhood and traveller's diarrhoea while EPEC has been associated with watery diarrhoea of infants (Mamun *et al*., 1993; Sang *et al*., 2012). The predominant bacterial cause of diarrhoea was EAEC in our study. These results differ with Sang *et al.* (2012) findings where EPEC was reported as the common bacterial pathogen in diarrhoea in four provinces in Kenya. This could be attributed to the effectiveness of introduction of zinc supplementation plus oral rehydration salts to improve diarrhoea management in children (Bhutta *et al.,* 1999; WHO, 2005; Shah *et al.,* 2012) as well as the encouragement of 0-6 months exclusive breastfeeding which have been reported to decreases morbidity from gastrointestinal diseases (WHO, 2013).

The interval between onset of symptoms and collection of specimens was 1-3 days for all patients where ETEC and EPEC were isolated. This is in agreement with other studies where ETEC and EPEC infections have been reported to have an abrupt onset with a short incubation period; 14 to 50 hours for ETEC and 2.9 hours for EPEC (Donnenberg *et al.*, 1993; Nataro & Kaper, 1998). This leads to rapid search for medical intervention. Our results seem to support the association of EAEC with acute diarrhea disease. All the EAEC infected patients presented to the health facility within 7 days of diarrhea onset suggestive of an acute infection. This compares with

a study conducted in Tanzania, where EAEC was the predominant diarrhoeagenic *E. coli* strain isolated in children under-5 years presenting with acute diarrhea (Jordi *et al.,* 1999). However, EAEC has also been associated persistent diarrhea among Kenyan children (Sang *et al*., 1997).

Contaminated drinking water has frequently been associated with acute onset of diarrhea disease. Our study observed a significant association between drinking borehole, un-treated and un-boiled water with DEC infection. Implementation of water, sanitation and hygiene (WASH) programmes have been reported to reduce diarrhoea diseases by interrupting faecal–oral transmission pathways, commonly referred to as the five "F" (fluids, fields, flies, fingers and food). These entail the provision of safe water, the use of sanitation facilities and hygiene education. Water storage practices also contribute to contamination of otherwise safe water (Fewtrell *et al.,* 2005; Onyango and Angienda, 2010; Wittenberg, 2012).

5.2 Antimicrobial susceptibility patterns

High prevalence of resistance to commonly used antimicrobials such as ampicillin and trimethoprim /sulfamethoxazole was observed (Figure 4.2). This is consistent with other studies in different parts of Kenya which have reported high ampicillin and trimethoprim /sulfamethoxazole resistance among enteric bacterial pathogens (Sang *et al.,* 2012; Sang *et al.,* 2011). Use of expanded-spectrum cephalosporins (ceftazidime, ceftriaxone or cefotaxime) or aztreonam in antimicrobial susceptibility test serves to test presence of Extended Spectrum β-Lactamase (ESBL)-Producing *Enterobacteriaceae* (Mark and Paul, 2003; CLSI, 2016).

ESBLs producing micro-organisms have hydrolytic and inactivating effect on oxyimino-aminothiazolyl cephalosporins such as cefuroxime, cefotaxime, ceftriaxone, ceftizoxime, ceftazidime, cefpirome and cefepime, as well as penicillins, aztreonam and other cephalosporins with the exception of cephamycins (cefoxitin) leading to their resistance (Henquell *et al*., 1995; Odonkor and Addo, 2011; Kiiru *et al.,* 2012). These plasmid mediated enzymes are caused by mutations of TEM-1 and TEM-2 (Temoniera enzymes) and SHV-1 (Sulfhhdryl variable enzyme) (Odonkor

and Addo, 2011) and are commonly found in the *Enterobacteriaceae* family. The antimicrobial susceptibility patterns observed in our study are suggestive of presence of ESBLs. This is indicated by the resistance pattern of cephalosporins and cefoxitin (Kiiru *et al.,* 2012). Thus individuals infected by these resistant isolates now have a limited choice of treatment as none of the penicillins, cephalosporins or aztreonam antibiotics can effectively be used in treatment. These findings render ampicillin and trimethoprim /sulfamethoxazole, the recommended drugs of choice for empirical diarrhea treatment, as ineffective.

All DEC isolates were sensitive to ciprofloxacin and gentamicin. Common enteric pathogen, *Cholera, Salmonella, Shigella* and diarrhoeagenic *E. coli* have been found to be sensitive to Ciprofloxacin. In 2010, a study conducted among the urban refugees in Kenya reported complete ciprofloxacin sensitivity by *Salmonella, Shigella* and ETEC isolates (Waqo *et al*., 2013). In 2011, Sang *et al.* (2011) reported similar findings among the Maasai community. A Tanzanian study also reported complete sensitivity to ciprofloxacin (Jordi *et al*., 1999). Similarly, Gentamicin has also shown absolute sensitivity to DEC, *Vibrio cholerae* and *Shigella* isolated from different parts of Kenya (Sang *et al*., 2012; Waqo *et al.*, 2013). However, low resistance levels (1-5%) to ciprofloxacin and gentamicin have been reported across the country (Sang *et al*., 2011, Sang *et al*., 2012, Waqo *et al.*, 2013). Additionally, carbapenems and cephamycins were effective against these isolates. This corresponds to Kiiru *et al,* (2012) findings among *E. coli* isolates obtained from patients in Kenya for a period of 18-years. These findings are suggestive of gentamicin and ciprofloxacin being the drug of choice where empirical diarrhea treatment is observed. Nevertheless, systematic clinical laboratory base antimicrobial surveillance is needed to guide optimal diarrhea case management, antimicrobial stewardship and frequent updating of treatment guidelines not only at institutional levels (e.g. referral and private hospitals) but also at regional or national levels.

5.3 Limitations of the study

This study was a hospital based study. This theoretically limits representativeness and generalizability. Despite this limitation, the study documents the existence of DEC among patients seeking diarrhea treatment in Thika District Hospital.

CHAPTER SIX

CONCLUSION AND RECOMMEDATIONS

6.1 Conclusion

1) The period prevalence of diarrhoeagenic E. coli was 17.9% (72).

2) Three (3) DEC strains; 60 (83.3%) EAEC, 6 (8.3%) EPEC and 6 (8.3%) ETEC, were isolated. EAEC were the most predominant pathotypes.

3) High resistance proportion (92%) to commonly used antimicrobials; ampicillin and trimethoprim /sulfamethoxazole, for empirical diarrhea treatment were observed.

4) Low levels (15%) of antimicrobial resistance to cephalosporins and aztreonam were observed.

6.2 Recommendations

1) Molecular characterization of *E. coli* isolates is necessary to establish their pathotypes.

2) Systematic clinical antimicrobial surveillance to guide choice of treatment options for optimal diarrhea management and antimicrobial stewardship should be observed.

3) In-effective antimicrobials should be removed from the treatment guidelines and withdrawn from the market.

4) Hygiene education especially effective hand washing technique in primary and secondary schools.

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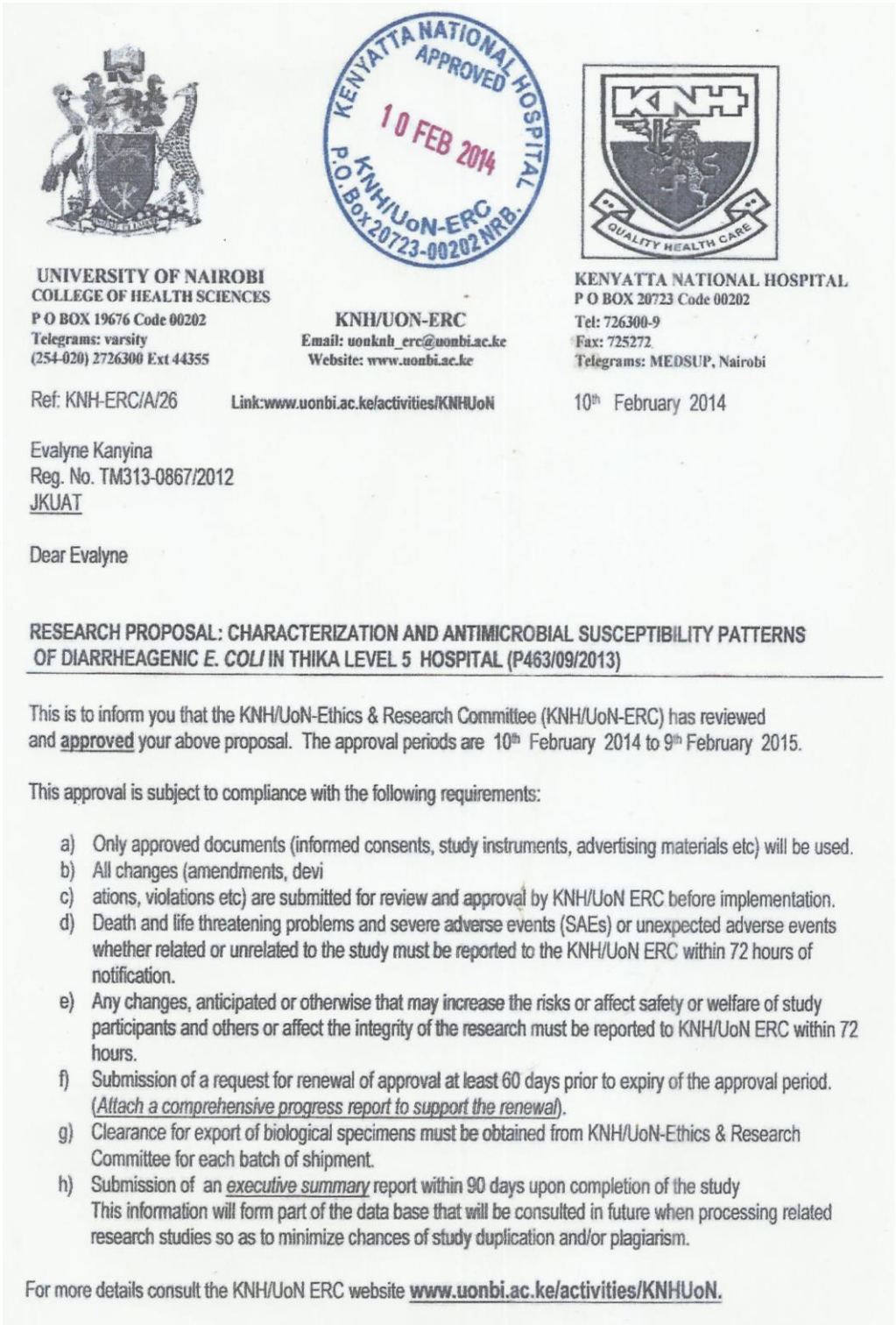
APPENDICES

Appendix 1: KHN-UoN Ethics and Research Committee (ERC) approval



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Evalyne Kanyina
Reg. No. TM313-0867/2012
JKUAT

Dear Evalyne

RESEARCH PROPOSAL: CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF DIARRHEAGENIC *E. COLI* IN THIKA LEVEL 5 HOSPITAL (P463/09/2013)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 10th February 2014 to 9th February 2015.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, devi
c) ations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
d) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
e) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
f) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
g) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
h) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN.**

Appendix 2: Thika Level 5 Hospital Research and Ethics Committee (ERC) approval

MINISTRY OF HEALTH

Tel.Thika 067 21621/2 fax 21778
All correspondence should be addressed to MED.SUPT.
When replying please quote

Ref; NO. MOH/TKA/

THIKA LEVEL 5 HOSPITAL
P.O. BOX 227
THIKA

Date: 27th March,2014

To Jackline Wambui

REF: RESEARCH APPROVAL

Title: **CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF DIARRHEAGENIC E.COLI IN THIKA LEVEL 5 HOSPITAL (P463/09/2013)**

Having discussed your research proposal, the Thika Level 5 Hospital research and ethics committee hereby gives you the green light to conduct above research after you clear the requisite fees.
You are adviced to strictly adhere to the data collection period as you outlined in the proposal. Request for extra data collection time must be made to the committee in writing. You are further advised to strictly stick to research ethics and staff and patients/clients confidentiality must not be breached.
Any data or information you may come across which does not form part of your research must not be used/ broadcast/divulged to other people without express authority of the hospital Medical Superintendent.

As you conduct your research you will be attached to Dr. Muthami who is the head of department where you will be conducting the research.

On completion of the research you are expected and required to inform the hospital of your findings. This gives you an opportunity to help improving the provision of quality heath care at Thika Level 5 hospital.

In case you are found to contravene or violate the code of ethics the hospital reserves the right to terminate your research without prior warning.

We look forward to the findings of the research and we wish you the best.

MEDICAL SUPERINTENDENT 27 MAR 2014 THIKA LEVEL 5 HOSPITAL

Thank you.

DR. MBOGO

I..... Agree and will adhere to the above terms.
Signed.....
Date..... 3rd April 2014

1 | Page

Appendix 3: Survey questionnaire

Research Topic: Characterization and Antimicrobial Susceptibility Pattern of Diarrheagenic *E. coli* in Thika level 5 Hospital

1) Date of interview Day [ ] Month [ ] Year [ ]
2) Participant number [ ]
3) Participant date of birth Day [ ] Month [ ] Year [ ]
4) Participant age [ ]
5) Gender: Male [ ] Female [ ]
6) What is the highest level of education you have attained

i) None [ ] ii) Primary [ ]
iii) Secondary [ ] iv) College (post-secondary) [ ]

7) What is the source of your drinking water?

i) Piped in the house [ ] ii) Piped into a common water point [ ]
iii) Bore hole [ ] iv) River / spring [ ]
iv) Others (explain) _____

8) Which containers do you use for water storage?

i) Buckets [ ] ii) Jerry cans (Kibuyu) [ ]
iii) Tank [ ] iv) Drums [ ]
v) Others. Specify……

9) What is the size of the mouth of your water storage container?

i) Big [ ] ii) Narrow [ ]

10) Do you boil your water?

i) Yes [ ] ii) No [ ]

11) Do you treat your water?

i) Yes [ ] ii) No [ ]

12) Which of this describe your toilet?

i) Built into the house [ ] ii) Serving members of the whole plot [ ]

iii) Others (explain) _____

13) When did you start having this diarrhea? Day [ ] Month [ ] Year [ ]

i) 1-3 days ago [ ] ii) 4-6 days ago

iii) >6days ago [ ]

14) Have you taken any medications for diarrhea in the past 72hrs (3 days)?

i) Yes [ ] ii) No [ ]

If yes, list medication taken. ……

If No, skip to question 15.

15) Where was medication for diarrhea obtained?

i) Shop [ ]

ii) Chemist [ ]

iii) Health Institution [ ]

iv) Herbalist [ ]

v) Homemade [ ]

vi) Others [ ]

explain ……

16) Which of these signs and symptoms are you experiencing?

i) Stomach ache [ ] ii) Headache [ ]

iii) Vomiting [ ] iv) Fever [ ]

v) Others (explain)

END THE INTERVIEW

Appendix 4: Study participation consent form

Study Title: Characterization and antimicrobial susceptibility pattern of diarrheagenic *E. coli* in Thika Level 5 Hospital

Investigator

Evalyne Kanyina. BSC Medical Laboratory Science, MSc Laboratory Management and Epidemiology, **E. mail:** jkanyina@yahoo.com

Emergency telephone number: Principal Investigator Mobile: 0723 36 72 05

Kenyatta National Hospital/University of Nairobi - Ethics & Research Committee

Telephone number : +2542726300-19 Ext.44102

E-mail: knhuonerc@gmail.com Post address: P O BOX 20723-00202, Nairobi, Kenya.

Investigator's statement: I am requesting you to be in the study. The purpose of this consent form is to give you the information you will need to help you decide whether to be in the study or not. Please read this form carefully or listen as it is read to you. You may ask questions about what we will ask you to do, the risks, the benefits and your rights as a volunteer, or anything about the research or in this form that is not clear. When all your questions have been answered, you can decide if you want to be in the study or not. This process is called "informed consent" – a knowledgeable agreement. You are free to refuse to participate and to withdraw from the study at any time without penalty or loss of benefits.

Purpose and benefits: The aim of this study is to determine the distribution and drugs performance patterns of diarrhea causing *E. coli* among patients presenting with diarrhea in Thika Level 5 Hospital. The study will inform diarrhea management as well as give you the results of test done to you. You can take part in the study if you have come for stool culture and sensitivity test.

Procedures: This is what will happen if you decide to participate in this study. The researcher will ask you several questions regarding you. After which you will be informed on how you will collect a stool sample and bring it to the laboratory for

analysis in a stool collecting container. The specimens will be marked with a study number and not your name making it impossible to trace the results back to you. The specimen will be examined for culture and sensitivity and thereafter for specific disease causing agents. You will be informed of the initial findings after 3 days and guided for treated according to Thika Level 5 Hospital guidelines.

Risks, stress, or discomfort: You will not be subjected to any other sample collection procedure for the purpose of the study after stool collection. In case your stool sample needs to be collected by a rectal swab, you might fell embarrassed and some discomfort which will take a short time to end. The rectal swab stool collection procedure involves gently inserting a sterile cotton swab 1-1.5 inches into the study participant anus and gently rotating it to facilitate stool sample collection. Participation in the study will require you to commit your time. Completing the questions will take 5-10 minutes. However, we will try to serve you as quick as possible.

Reimbursement: You will not receive any money for involvement in this survey.

Other information: we will keep your identification as a research participant confidential. The information about you will be identified only by the study number and will not be linked to your name in any record. You are free to refuse to participate in the study, if you decide not to participate in the study you will receive similar care to that provided to others participating in the study.

Participant signature /thumb print…… Date ……
Witness signature/ thumb print…….. Date …….

Appendix 5: Study participation consent form (Kiswahili Version)

Utafiti Kichwa: usambazaji wa ugonjwa wa kuhara unaosababishwa na viini za diarrheagenic E. coli na tabia za dawa dhidi ya hizi viini katika Hospitali ya Thika Level 5

Mpelelezi mkuu:

Evalyne Kanyina. Barua pepe: jkanyina@yahoo.com

Namba ya simu: 0723 36 72 05

Namba ya simu ya dharura

Simu ya mpelelezi mkuu: 0723 36 72 05

Hospitali la Kitaifa la Kenyatta / Chuo Kikuu cha Nairobi –

Namba ya simu ya maadili ya Utafiti wa Kamati: 25 42 72 63 00-19 Ext.44102

Barua pepe: knhuonerc@gmail.com

Neno kutoka kwa mpelelezi mkuu:

Ninaomba wewe kuwa katika utafiti huu. madhumuni ya fomu hii ni kukupa habari unahitaji kukusaidia kuamua kama kuwa katika utafiti huu au la. Tafadhali soma fomu hii kwa makini au usikilize kama na kusomea. Unaweza kuuliza maswali kuhusu huu utafiti tunao tekeleza, hatari, faida na haki zako kama mtu wakujitolea, au kitu chochote kuhusu utafiti au katika fomu hii. Wakati maswali yako yote yamejibiwa, unaweza kuamua kuwa katika utafiti huu au la. Mchakato huu unaitwa 'ridhaa'. Wewe ni huru kukataa kushiriki au kuomba kujiondoa kutoka utafiti wakati wowote bila ya adhabu au hasara ya faida.

Lengo la utafiti: kuamua usambazaji na madawa ya utendaji chati ya kuhara kunako sababishwa na viini vya E. coli kati ya wagonjwa wanao hara katika Hospitali ya Thika Level 5.

Taratibu kujishajilisha: Unaweza kuchukua sehemu katika utafiti huu kama una kuja kwa ajili ya kupimwa kinyesi (choo kukubwa). Mtafiti atauliza wewe maswali kadhaa kujihusu. Baadaye, atakupa taarifa juu ya namna ya kukusanya sampuli ya kinyesi na kuleta kwa maabara kwa ajili ya uchambuzi wa kinyesi. Nambari zitatumika kama vielelezo walasi jina lake ili kuifanya vigumu kufuatilia matokeo yako. Taarifa ya matokeo ya utafiti itatolewa baada ya siku 3 kwamhusika ili kuongoza kwa matiabu kulingana na miongozo ya hospitali la Thika Level 5.

Hatari, dhiki, au usumbufu: Hakuna utaratibu mwingine wa ukusanyaji wa sampuli kwa madhumuni ya utafiti baada ya ukusanyaji wa kinyesi. Kama kinyesi chako kitatolewa kwa njia ya rectal swab, unaweza akaanguka aibu na baadhi ya uchungu ambao itachukua muda mfupi. Utaratibu wa rectal swab unahushisha kuingiza kwa upole kijiti cha hiyo swab 1-1.5 inches ndani ya njia ya choo kubwa na kuizungusha pole pole ilikuwezeshe kukushanya kinyesi.

Ushiriki katika utafiti itamgarimu muda wako ili kukamilisha maswali yatakayochukua muda wa dakika tano ama kuni. Hata hivyo, sisi tutajaribu kumtumikia haraka iwezekanavyo.

Malipo: Hutapokea fedha zozote kwa ajili ya ushiriki katika utafiti huu.

Taarifa nyinginezo: Kitambulisho chako ama jina lakukutambulisha kama mshiriki wa utafiti itawekwa kama siri. taarifa yako itatabuliwa kwa nambari ya utafiti na si kwa jina lako katika rekodi zozote. Wewe unaweza kukataa kushiriki katika utafiti nautapata huduma sawa zinazotolewa kwa wengine wanaoshiriki katika utafiti.

Sahihi ya mshirika wa utafiti…..……..... Tarehe ……

Appendix 6: Study participation assent form

Study Title

Characterization and antimicrobial susceptibility pattern of diarrheagenic *E. coli* in Thika Level 5 Hospital

Investigator

Evalyne Kanyina. BSC Medical Laboratory Science, MSc Laboratory Management and Epidemiology, **E. mail:** jkanyina@yahoo.com

Emergency telephone number: Principal Investigator Mobile: 0723 36 72 05

Kenyatta National Hospital/University of Nairobi - Ethics & Research Committee

Telephone number: +2542726300-19 Ext.44102, E-mail: knhuonerc@gmail.com

Post address: P O BOX 20723-00202, Nairobi, Kenya.

Investigator's statement:

I am requesting your child/relative to be in the study. The purpose of this assent form is to give you the information you will need to help you decide whether to allow her to be in the study or not. Please read this form carefully or listen as it is read to you. You may ask questions about what we will ask him/her to do, the risks, the benefits and her rights as a volunteer, or anything about the research or in this form that is not clear. When all your questions have been answered, you can decide if you will allow her to be in the study or not. This process is called "informed consent." You are free to refuse her participation or request her withdrawal from the study at any time without penalty or loss of benefits.

Purpose and benefits: The aim of this study is to determine the distribution and drugs performance patterns of diarrhea causing *E. coli* among patients presenting with diarrhea in Thika Level 5 Hospital. The study will inform diarrhea management as well as give you the results of test done to you. You can take part in the study if you have come for stool culture and sensitivity test.

Procedures: This is what will happen if you allow her/him to participate in this

study. The researcher will ask (s)he several questions regarding him/herself. After which (s)he will be informed on how they will collect a stool sample and bring it to the laboratory for analysis in a stool collecting container. The specimens will be marked with a study number and not his/her name making it impossible to trace the results back to him/her. The specimen will be examined for culture and sensitivity and thereafter for specific disease causing agents. (S)he will be informed of the initial findings after 3 days and guided for treated according to Thika Level 5 Hospital guidelines.

Risks, stress, or discomfort: (S)he will not be subjected to any other sample collection procedure for the purpose of the study after stool collection. In case his (her) stool sample needs to be collected by a rectal swab, (s)he might fell embarrassed and some discomfort which will take a short time to end. The rectal swab stool collection procedure involves gently inserting a sterile cotton swab 1-1.5 inches into the study participant anus and gently rotating it to facilitate stool sample collection. Participation in the study will require his/her to commit his/her time. Completing the questions will take 5-10 minutes. However, we will try to serve him/her as quick as possible.

Reimbursement: (S)he will not receive any money for involvement in this survey.

Other information: we will keep his/her identification as a research participant confidential. The information about him/her will be identified only by the study number and will not be linked to his/her name in any record. You or (S)he are free to refuse participating in the study, if (s)he decide not to participate in the study he/she will receive similar care to that provided to others participating in the study.

Participant signature/ Name…… Date …….

Witness signature/ Name……. Date …….

Appendix 7: Study participation assent form (Kiswahili Version)

Utafiti Kichwa: usambazaji wa ugonjwa wa kuhara unaosababishwa na viini za diarrheagenic E. coli na tabia za dawa dhidi ya hizi viini katika Hospitali ya Thika Level 5

Mpelelezi mkuu:

Evalyne Kanyina. Barua pepe: jkanyina@yahoo.com

Namba ya simu: 0723 36 72 05

Namba ya simu ya dharura

Simu ya mpelelezi mkuu: 0723 36 72 05

Hospitali la Kitaifa la Kenyatta / Chuo Kikuu cha Nairobi –

Namba ya simu ya maadili ya Utafiti wa Kamati: 25 42 72 63 00-19 Ext.44102

Barua pepe: knhuonerc@gmail.com

Neno kutoka kwa mpelelezi mkuu:

Ninaomba mtoto wako /jamaa kuwa katika utafiti huu. madhumuni ya fomu hii ni kukupa habari unahitaji kukusaidia kuamua kama kumruhusu yeye kuwa katika utafiti huu au la. Tafadhali soma fomu hii kwa makini au usikilize kama na kusomea. Unaweza kuuliza maswali kuhusu huu utafiti tunao tekeleza, hatari, faida na haki zako kama mtu wakujitolea, au kitu chochote kuhusu utafiti au katika fomu hii. Wakati maswali yako yote yamejibiwa, unaweza kuamua kumruhusu kuwa katika utafiti huu au la. Mchakato huu unaitwa 'ridhaa'. Wewe ni huru kukataa kumshirikisha au kuomba kujiondoa kutoka utafiti wakati wowote bila ya adhabu au hasara ya faida.

Lengo la utafiti: kuamua usambazaji na madawa ya utendaji chati ya kuhara kunako sababishwa na viini vya E. coli kati ya wagonjwa wanao hara katika Hospitali ya Thika Level 5.

Taratibu kujishajilisha: Unaweza kuchukua sehemu katika utafiti huu kama una kuja kwa ajili ya kupimwa kinyesi (choo kukubwa). Mtafiti atamuliza yeye/wewe maswali kadhaa kujihusu/kumuhusu. Baadaye, atakupa taarifa juu ya namna ya kukusanya sampuli ya kinyesi na kuleta kwa maabara kwa ajili ya uchambuzi wa kinyesi. Nambari zitatumika kama vielelezo walasi jina lake ili kuifanya vigumu kufuatilia matokeo yake. Taarifa ya matokeo ya utafiti itatolewa baada ya siku 3 kwamhusika ili kuongoza kwa matiabu kulingana na miongozo ya hospitali la Thika Level 5.

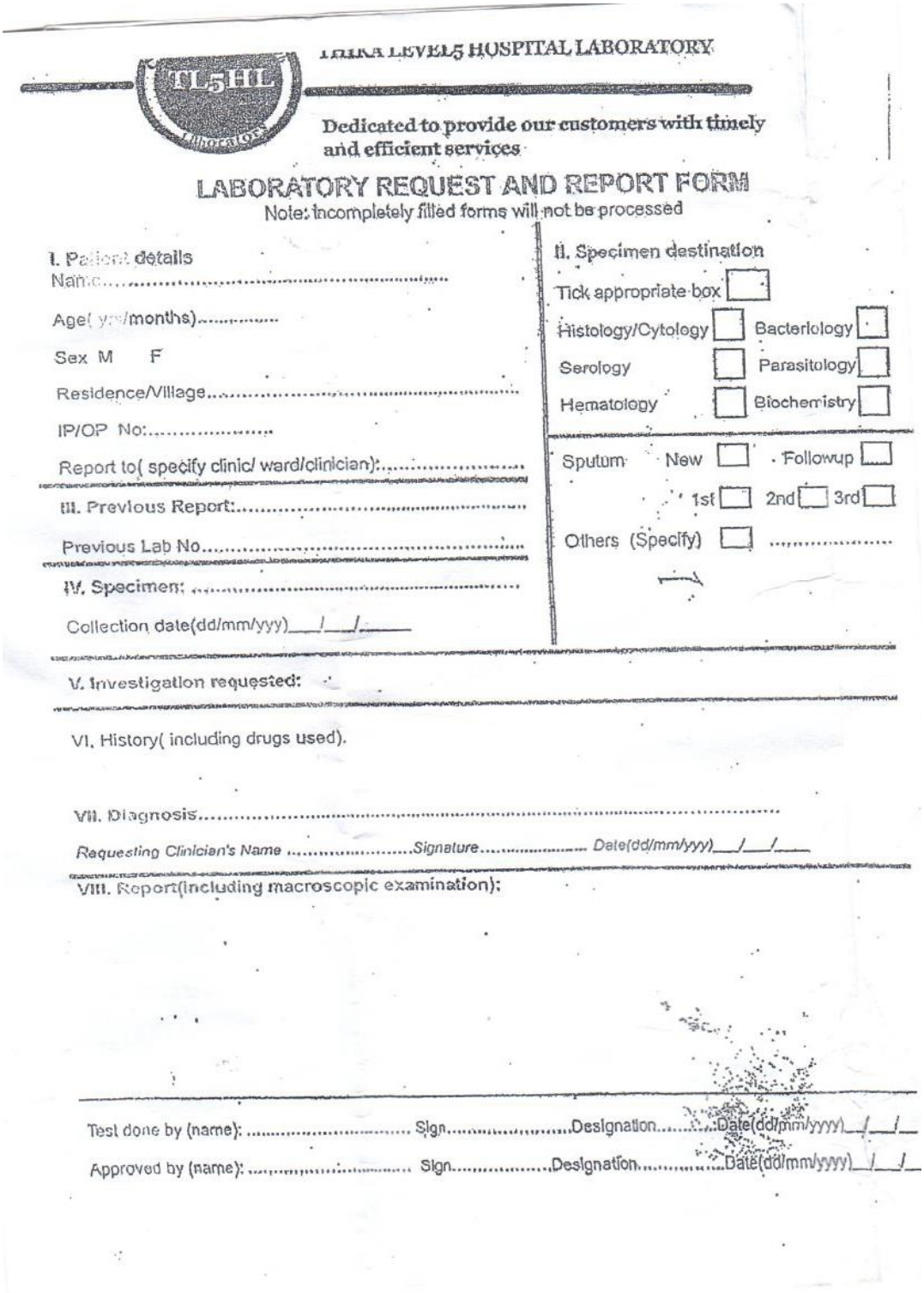
Hatari, dhiki, au usumbufu: Hakuna utaratibu mwingine wa ukusanyaji wa sampuli kwa madhumuni ya utafiti baada ya ukusanyaji wa kinyesi. Kama kinyesi chake kitatolewa kwa njia ya rectal swab, anaweza akaanguka aibu na baadhi ya uchungu ambao utachukua muda mfupi. Utaratibu wa rectal swab unahushisha kuingiza kwa upole kijiti cha hiyo swab 1-1.5 inches ndani ya njia yake ya choo kubwa na kuizungusha pole pole ilikuwezeshe kukushanya kinyesi. Ushiriki katika utafiti itamgarimu muda wake/wako ili kukamilisha maswali yatakayochukua muda wa dakika tano ama kuni. Hata hivyo, sisi tutajaribu kumtumikia haraka iwezekanavyo.

Malipo: Hutapokea fedha zozote kwa ajili ya ushiriki katika utafiti huu.

Taarifa nyinginezo: Kitambulisho chako ama jina lakukutambulisha kama mshiriki wa utafiti itawekwa kama siri. taarifa yako itatabuliwa kwa nambari ya utafiti na si kwa jina lako katika rekodi zozote. Wewe unaweza kukataa kushiriki katika utafiti nautapata huduma sawa zinazotolewa kwa wengine wanaoshiriki katika utafiti.

Sahihi ya mshirika wa utafiti…..…….... Tarehe …….

Appendix 8: Laboratory request form



THIKA LEVEL5 HOSPITAL LABORATORY

TL5HL

Dedicated to provide our customers with timely and efficient services

LABORATORY REQUEST AND REPORT FORM

Note: Incompletely filled forms will not be processed

I. Patient details

Name.....

Age(yrs/months).....

Sex M F

Residence/Village.....

IP/OP No:.....

Report to(specify clinic/ ward/clinician):.....

II. Specimen destination

Tick appropriate box

Histology/Cytology ☐ Bacteriology ☐

Serology ☐ Parasitology ☐

Hematology ☐ Biochemistry ☐

Sputum New ☐ Followup ☐

1st ☐ 2nd ☐ 3rd ☐

Others (Specify) ☐ .....

III. Previous Report:.....

Previous Lab No.....

IV. Specimen: .....

Collection date(dd/mm/yyy)__/__/____

V. Investigation requested:

VI. History(including drugs used).

VII. Diagnosis.....

Requesting Clinician's Name .....Signature..... Date(dd/mm/yyy)__/__/____

VIII. Report(including macroscopic examination):

Test done by (name): ..... Sign.....Designation.....Date(dd/mm/yyyy)__/__/__

Approved by (name): ..... Sign.....Designation.....Date(dd/mm/yyyy)__/__/__

Appendix 9: VITEK Antimicrobial Susceptibility Test (AST) material data sheet

VITEK 2 — technology

AST-P586

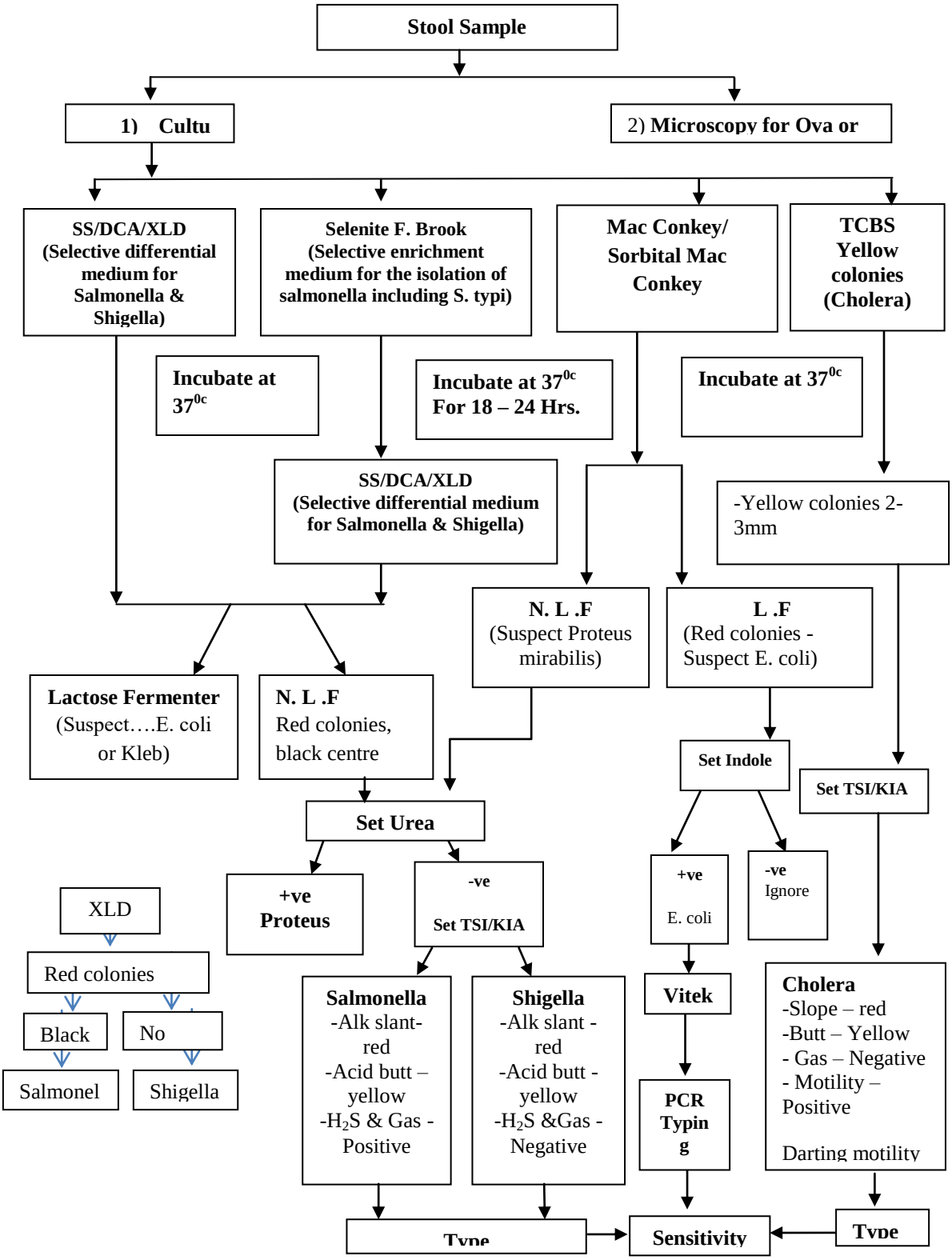
REF 22 276

8°C

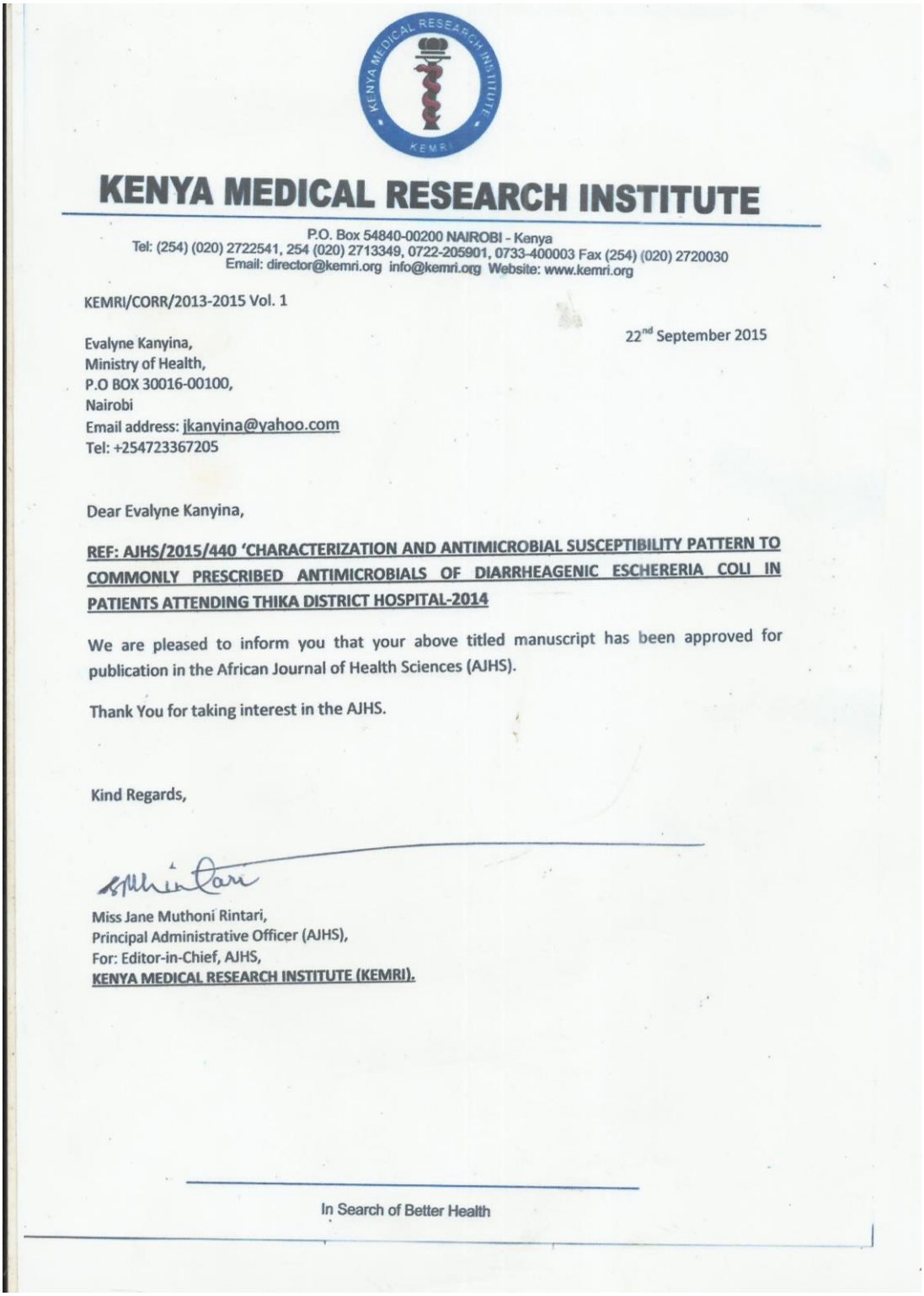
IVD

For In Vitro Diagnostic

Appendix 10: Laboratory analysis flow chart



Appendix 11: Acceptance for manuscript publication



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/CORR/2013-2015 Vol. 1

22nd September 2015

Evalyne Kanyina,
Ministry of Health,
P.O BOX 30016-00100,
Nairobi
Email address: jkanyina@yahoo.com
Tel: +254723367205

Dear Evalyne Kanyina,

REF: AJHS/2015/440 'CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERN TO COMMONLY PRESCRIBED ANTIMICROBIALS OF DIARRHEAGENIC ESCHERERIA COLI IN PATIENTS ATTENDING THIKA DISTRICT HOSPITAL-2014

We are pleased to inform you that your above titled manuscript has been approved for publication in the African Journal of Health Sciences (AJHS).

Thank You for taking interest in the AJHS.

Kind Regards,

Miss Jane Muthoni Rintari,
Principal Administrative Officer (AJHS),
For: Editor-in-Chief, AJHS,
KENYA MEDICAL RESEARCH INSTITUTE (KEMRI).

In Search of Better Health

Appendix 12: Published abstract

Characterization and Antimicrobial Susceptibility Pattern to Commonly Prescribed Antimicrobials of Diarrheagenic Escherichia coli in Patients attending Thika District Hospital – Kenya, 2014.

Kanyina E, Sang W, Kiiyukia C, Tonui J, Boru W, Galgalo T
Kanyina E[1, 2, 3, 4], Sang W[5], Kiiyukia C[4], Tonui J[1], Boru W[1, 3], Galgalo T[2, 6]

1. Ministry of Health,Nairobi, Kenya.
2. East Africa Public Health Laboratory Networking Project, Ministry of Health.
3. Field Epidemiology and Laboratory Training Program.
4. Institute of Tropical Medicine, Jomo Kenyatta University of Agriculture and Technology, College of Health Science.
5. Center for Microbiology Research Laboratory, Kenya Medical Research Institute, Nairobi, Kenya.
6. African Field Epidemiology Network, Kampala, Uganda

Corresponding Author: Evalyne Kanyina. E-mail: jkanyina@yahoo.com, Tel: +254 723 367 205

Summary

Background: Diarrheagenic *E. coli* (DEC) are associated with outbreaks of severe diarrhea and multiple drug resistance. We characterize DEC among diarrhoeal patients attending Thika Hospital and determine their antimicrobial susceptibility patterns.

Methods: A cross-sectional study was conducted among patients of all ages seeking diarrhea treatment. Stool samples were collected, inoculated on bacterial differential media for growth of enteric pathogens, characterized and antimicrobial susceptibility of DEC isolates determined.

Results: A total of 402 stool samples were cultured. *E. coli* was isolated from 269, of which 72 (27%) were DEC; 60 (83.3%) enteroaggregative *E. coli* (EAEC), 6 (8.3%) enteropathogenic *E. coli* (EPEC) and 6 (8.3%) enterotoxigenic *E. coli* (ETEC). Of the 72, 58% were female, median age was 8 (IQR: 2-28) years, 75% did not boil water and 100% did not treat water. Twenty five (35%) patients with DEC were under-five years. Drinking un-boiled water (OR: 2.51, 95% CI: 1.36-4.61) was associated with having DEC. All DEC isolates were sensitive to cefoxitin, meropenem, amikacin, gentamicin and ciprofloxacin. They were resistance to ampicillin (92%), trimethoprim-sulfamethoxazole (92%) and amoxicillin-clavulanic acid (85%).

Conclusion: The predominant DEC strain was EAEC. High resistant to ampicillin, trimethoprim-sulfamethoxazole and amoxicillin-clavulanic acid were observed. All isolates were sensitive to ciprofloxacin and gentamicin.

Key words: Diarrhea, *E. coli*, Diarrheagenic *E. coli*, Characterization, Kenya

[Afr J Health Sci. 2016; 29(1):25-35]

Appendix 13: Conference poster presentation

Characterization and Antimicrobial Susceptibility Pattern to Commonly Prescribed Antimicrobials of Diarrheagenic Escherichia coli in Patients attending Thika District Hospital - Kenya, 2014

Evalyne Kanyina[1, 2, 3], Willie Sang[4], Ciira Kiiyukia[5], Waqo Boru[1, 2], Tura Galgalo[1, 6]

1) Kenya Field Epidemiology and Laboratory Training Programme, Kenya. 2) Ministry of Health, Kenya. 3) East African Public Health Laboratory Network Project, Kenya. 4)Center for Microbiology Research Laboratory, Kenya Medical Research Institute, Kenya. 5) Institute of Tropical Medicine, Jomo Kenyatta University of Agriculture and Technology, College of Health Science, Kenya.6) Africa Field Epidemiology Network, Kenya.

Introduction

- Diarrheagenic E. coli (DEC) are associated with outbreaks of severe diarrhea and multiple drug resistance
- Transmitted through consumption of contaminated water or food
- Accounts for a third of diarrheal episodes among children under-five years in low income countries
- Emergence of new virulent enteric pathogen and the emerging antibiotic resistance threatens effective treatment and management of diarrheal infections

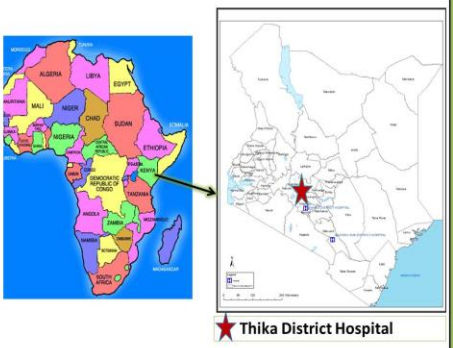
Objective

- To isolate, characterize DEC among diarrheal patients in Thika District Hospital
- Determine their antimicrobial susceptibility pattern
- Determine DEC association with socio-demographic

Methods

Study sites and time period

Thika District Hospital in Kenya; April to July 2014



Case definition

Consenting and assenting patients of all ages seeking diarrhea treatment at the hospital from April to July 2014

Study design

- A hospital based cross-sectional study

Data collection

- Cases interviewed using structured questionnaire to collect clinical and epidemiologic information

Laboratory analysis

- Stool samples collected, inoculated on bacterial differential media for growth of enteric pathogens
- Bacteria identified morphologically and biochemically
- Characterized by Polymerase Chain Reaction (PCR)
- Antimicrobial susceptibility of DEC isolates determined

Data analysis

- Data entered and analyzed using Epi info version 3.5.1.

Ethical approval

- Kenyatta National Hospital ethical review committee

Results

Table 1: Demographics characteristic of diarrhea cases, Thika District Hospital, April to July, 2014

Variable	Freq (%)	Variable	Freq (%)
Gender	222 (55%) female	Level of education	
Median age (years)	14 (Range: 1-77)	No formal education	6 (2%)
Cases under 5 years	133 (33%)	Primary	114 (28%)
Drinking borehole water	73 (18%)	Secondary	204 (51%)
Drinking piped water	329 (82%)	Post-secondary	78 (19%)
Drinking un-boiled water	246 (61%)	Communal toilet	270 (67%)

Total diarrhea cases
402

Organism isolated, n= 301		Isolated DEC, n=72		DEC drug resistance, n=72	
- Escherichia coli	269 (89%)	- EAEC	60 (83.3%)	- Ampicillin	66 (92%)
- DEC	72 (27%)	- EPEC	6 (8.3%)	- Cotrimoxazole	66 (92%)
- Pseudomonas spp	13 (4.3%)	- EHEC	6 (8.3%)	- Amoxicillin-clavulanic acid	61 (85%)
- Citrobacter spp	12 (4%)			- Ciprofloxacin	0 (0%)
- Klebsiella spp	6 (2%)			- Gentamicin	0 (0%)
- Salmonella spp	1(0.3%)				

Figure1: Summary of bacterial pathogen isolated among diarrhea patients in Thika District Hospital, April to July, 2014

Table 2: Bivariate analysis of factors associated with Diarrheagenic *E. coli* among diarrhea patients in Thika District Hospital, April to July, 2014

Variable	Odds Ratio	95% Confidence interval
No post secondary	3.45	1.40 - 8.46
Drinking borehole water	2.26	1.16 - 4.38
Drinking un-boiled water	2.58	1.41 - 4.70

Table 3: Multivariate analysis of factors associated with Diarrheagenic *E. coli* among diarrhea patients in Thika District Hospital, April to July, 2014

Variable	Adjusted Odds Ratio	95% Confidence interval
No post secondary	3.32	1.34 - 8.22
Drinking un-boiled water	2.51	1.36 - 4.61

Limitations

- Hospital based study limiting representativeness and generalizability

Recommendations

- Characterization of *E. coli* isolates in diarrhea patients to establish their pathotypes
- Systematic clinical antimicrobial surveillance to guide optimal diarrhea management and antimicrobial stewardship
- Health education on water, hygiene and sanitation in primary and secondary school

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